THE IMMUNIZING ANTIGEN OF THE PNEUMOCOCCUS.

III. The Purification of the Water-Soluble Antigen.

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(Received for publication, August 13, 1925.)

It has been shown by Perlzweig and Steffen (1) that the immunizing antigen of the pneumococcus is easily soluble in and extracted by water and salt solutions. This observation was later corroborated by Ferry and Fisher (2). It appeared desirable, therefore, to attempt to extract the antigen from the filtrates of broth cultures of pneumococcus.

Source of the Material.

The material used was obtained in the following manner. Massive cultures of Pneumococcus Type I were grown in the medium recently described by Huntoon (3) consisting of aminoids, peptone, sodium chloride, phosphates, and lactose. At the end of 18 hours of growth the culture was killed by heating at 56–60°C. for 1 hour, and centrifugalized through a Sharples machine. The supernatant fluid was passed through Mandler filters and then concentrated under reduced pressure at 40–44°C. to about one-fourteenth of the original volume.[1] This concentrated material was preserved with 0.5 per cent chloroform and kept in the refrigerator. On cooling, a large amount of lactose and the salts of the culture medium sedimented out. In the experiments described below the supernatant fluid only was used, as it was found that the sediment contained no appreciable quantity of the active material.

Purification by Pressure Filtration.

Since the original solution contained large amounts of salts, amino acids, lactose, peptones, and peptides it was first attempted to separate these by dialysis. The

[1] The growth of the cultures and the carrying out of the above preliminary treatment was done for us at the H. K. Mulford Laboratories at Glenolden Farms, Pa., under the supervision of Dr. Huntoon. The authors wish to express their appreciation to the Mulford Company and to Dr. Huntoon for their generous gift and cooperation.

osmotic pressure of the solution, however, proved to be too great, resulting in the passage of much water into the dialyzing bags, and thus materially retarding dialysis. Consequently it was found more advantageous to make use of ultrafiltration

TABLE I.

Experiment No	Fraction of antigen.	Total nitrogen per 100 cc.	Dose of antigen administered to mice.		Culture.	No. of mice.	
			1st dose.	2nd dose.		Dead.	Survived.
		mg.	*cc.*	*cc.*			
1 A	Original material including sediment.	1025	0.1	0.5	10^{-3}	3	1
					10^{-4}	1	3
					10^{-6}	1	2
1 B	Supernatant fluid of No. 1 A.	1005	0.1	0.5	10^{-3}	3	1
					10^{-6}	1	3
1 C	Ultrafiltration residue within 6 bags, obtained from No. 1 B.	32	0.1	0.5	10^{-3}	3	1
					10^{-6}	0	4
1 D	Ultrafiltrate of No. 1 C.	950	0.1	0.5	10^{-3}	4	0
					10^{-6}	4	0
2 A	Precipitate at pH 4.1 obtained from No. 1 C.	16	0.5		10^{-5}	0	8
2 B	Filtrate from No. 2 A.	16	0.5		10^{-5}	3	4
2 C	Hydrochloride of picrate precipitate from No. 1 C.	17	0.5		10^{-5}	1	7
					10^{-3}	1	0
Control (accompanying each of above experiments).					10^{-7}	2	0
					10^{-8}	2	0

under pressure. Stout collodion bags made from 8 to 12 per cent collodion in 1:1 alcohol-ether solution were used and found capable of withstanding pressures of 300 to 400 mm. of mercury. This method of ultrafiltration has been previously used with success by Glenny and Walpole (4) for the purification of diphtheria

antitoxin and of other similar substances. We did not, however, use water on the outside of the bags, but the filtrate was collected in glass jars without dilution. The material in the bags was washed for several days by continuous feeding into the bags of distilled water under pressure from a reservoir included in the system.

In this way it was possible to filter out through the membranes at least 97 per cent of the original content of nitrogen of our material (see Table I) and practically all of the inorganic salts. The total nitrogen content of the solution remaining within the bags was 32 mg. per 100 cc., whereas the original solution contained 1005 mg. per 100 cc. The dialysate gave positive protein color tests. It may be seen from the table that the content of immunizing antigen in this dialyzed fraction remaining in the bag compares well with that of the original solution.

Further Purification by Precipitation.

Further purification of the above dialyzed fraction was attempted by two methods: by precipitation of an active fraction at the isoelectric point and by the separation of a soluble picrate fraction. The first of these methods was proposed by Michaelis and Davidsohn (5) for the purification of diphtheria toxin, tuberculin, trypsin, etc., while the second was developed for the purification of insulin by Dudley (6) and applied by Baker, Dickens, and Dodds (7).

In a series of preliminary test-tube experiments it was found that a maximum sharply defined precipitation occurred in the solution of dialyzed material when a small amount of it was added to an excess of N/10 acetic acid-sodium acetate buffer mixture at pH 4.1.

Accordingly, to 100 cc. of the dialyzed solution 20 cc. of a N/2 acetate buffer mixture were added to pH 4.1. The precipitate was removed by decantation and centrifugalization and washed repeatedly in the centrifuge tube with diluted acetate mixture (pH 4.1). Finally, the precipitate was dissolved in water with the aid of a few drops of N/10 NaOH solution. This isoelectric fraction contained 20 mg. total nitrogen per 100 cc. calculated on the basis of the volume of the original solution. It protected mice against 1000 fatal doses of the homologous pneumococcus culture.

The picrate procedure was carried out according to the micro method of Baker, Dickens, and Dodds (7). 200 cc. of dialyzed solution were ground up in a mortar with 2.5 gm. of picric acid. The resulting precipitate was separated by centrifugalization and decantation, and washed several times in the centrifuge tube with saturated picric acid solution. The precipitate was then stirred in 70 per cent

acetone, whereupon much of it dissolved, yielding a dark brown-red solution and an insoluble bright yellow portion. The latter was separated in the centrifuge tube, and after decantation of the supernatant solution, the residue was extracted with two further portions of 70 per cent acetone. The supernatant acetone solutions were combined and evaporated to a small volume before a fan until a precipitate began to appear. On the addition of an equal volume, 10 cc. of water, a bulky, dark, gummy precipitate appeared. This was again separated in the centrifuge and washed repeatedly with ether to remove the excess of picric acid. The picrate was then changed into a hydrochloride by decomposing it with alcoholic HCl solution (25 cc. 3 N HCl diluted with 95 per cent alcohol to 100 cc.). All but a small portion dissolved. To the supernatant solution obtained on centrifuging 10 volumes of absolute acetone were added. A light flocculent precipitate separated out which darkened and became gummy on standing. This precipitate, representing the hydrochloride, was then removed by centrifugalization, washed with ether, and dried in a current of air. It was dissolved easily in very dilute $NaHCO_3$ solution. Before use in the protection experiments the pH of this solution was adjusted to 7.4. The hydrochloride fraction derived from the soluble picrate contained but 17 mg. of the 1005 mg. of total nitrogen in the original solution, and it also protected immunized mice against 1000 fatal doses of virulent Pneumococcus Type I culture.

The table given above shows the total nitrogen content of the various fractions calculated on the basis of the original solution. It also shows the results of the protection tests on mice. These were carried out according to the technique employed in the first paper of the series (8).

In some of the earlier experiments two doses of the protecting material were administered at 7 day intervals to the mice subcutaneously, while later it was found that one dose sufficed to produce immunity. For the purposes of infection a virulent 16 to 18 hour Pneumococcus Type I culture was given intraperitoneally 10 days after the last dose of antigen. Every experiment was controlled by at least two normal untreated mice which received intraperitoneally 0.5 cc. of 10^{-8} dilution of the same culture. This latter dose was adopted as the standard minimal fatal dose. Unless the control mice succumbed within 48 hours and pneumococci were recovered from a peritoneal smear or a subculture from the heart's blood, the experiment was discarded. All mice reported as dead succumbed within 60 hours and pneumococci were found in a stained peritoneal fluid smear. All mice reported as living or protected, survived at least 4 days and were in good condition at that time. In these experiments no effort was made to determine the maximal protecting power of the fractions.

DISCUSSION OF RESULTS AND SUMMARY.

It may be seen from the table that the soluble immunizing antigen which is present in the filtrate from cultures of the pneumococcus can be separated from non-antigenic material by means of ultrafiltration through collodion under pressure. It can be further purified by means of precipitation at a definite hydrion concentration and by the application of the picrate method used in the purification of insulin and of the antineuritic vitamine (9).

Whether the substance dealt with above corresponds to the protein obtained from bile solutions of pneumococci by Avery and Heidelberger (10) has not yet been established. This question is being studied along with others dealing with the chemical nature of the active antigen. Sufficient material for this purpose has been lacking until the present. The problem of species and type specificity of the various fractions is also to be investigated.

The immunologic experiments presented are to be regarded purely as qualitative tests to show relatively the absence or presence of some of the immunizing antigen in the fraction tested. No attempt has been made thus far to ascertain quantitatively the distribution of the antigen.

These methods obviously furnish a ready means for at least the initial separation and purification of the immunizing antigen from the complex mixtures present in culture media and in the bacterial cells themselves. Further chemical and immunologic studies will be made upon the fractions obtained by these methods.

SUMMARY.

Actively immunizing fractions of protein nature have been isolated from broth culture filtrates of Pneumococcus Type I by ultrafiltration, precipitation at a definite hydrion concentration, and the separation of a soluble picrate fraction. The method appears to be suitable for the initial purification of this antigen.

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3. Huntoon, F. M., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 268.
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TISSUE CHANGES FOLLOWING DEPRIVATION OF FAT-SOLUBLE A VITAMIN.

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Plates 37 to 40.

(Received for publication, September 4, 1925.)

INTRODUCTION.

No adequate account of the histopathology of experimental fat-soluble (vitamin) A deficiency has hitherto been published, and no conception of the pathogenesis of the striking gross effects observed in this condition has been possible.

Few pathological studies have been made, and the majority of these have resulted in wholly negative results and, therefore, erroneous conclusions as to the sequence of events and importance of infections.

Analysis of the following papers in most instances reveals as cause for failure either improperly composed diet or insufficient duration of the experiments. A number of workers have assumed that the eye and its glands alone deserved study—organs which we have found to exhibit much less striking lesions than the respiratory and genitourinary tracts and certain glands. In light of the pathology described in this paper it is no longer tenable to characterize the condition of fat-soluble A deficiency by names referring to the eye pathology; *i.e.*, xerophthalmia—keratomalacia. Atrophy of many glands, arrest of growth, emaciation, and replacement of many different epithelia by stratified keratinizing epithelium actually characterize fat-soluble A avitaminosis. The specific pathology is the widespread keratinization.

Emmett and Allen[1] in a comparison of changes due to vitamin A and B deficiency respectively in the rat report "no special outstanding pathological findings," in the absence of fat-soluble A, in contrast to atrophies and hypertrophies found in B deficiency. The duration of their experiments was not given.

Stephenson and Clark[2] failed to find a distinctive pathology in keratomalacia in rats.

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[1] Emmett, A. D., and Allen, F. P., *J. Biol. Chem.*, 1920, xli, p. liii.

[2] Stephenson, M., and Clark, A. B., *Biochem. J.*, 1920, xiv, 502.

Tsuji,[3] although his thesis was the effect of complete vitamin deficiency upon the thyroid, noted only atrophy of organs which he regarded as secondary to atrophy of the thyroid.

Davis and Outhouse[4] studied only the kidneys, spleen, heart, lungs, pancreas, liver, and testes. As they report that the testes were normal in most of their cases, it is certain that either their diet was not deficient in fat-soluble (vitamin) A or that the duration of the experiments was too short.

Cramer, Drew, and Mottram[5] in a study of the effects of vitamin deficiency in rats upon the function of lymphocytes and lymphoid tissue found no pathology in fat-soluble A deficiency. The duration of their experiments is not given.

Wason[6] found no lesions in any organ other than the eyes. She regarded the changes in the cornea as secondary to bacterial invasion.

Meyerstein[7] failed to find any characteristic pathology in either vitamin A or vitamin B deficiency in rats.

Outstanding papers dealing with the pathology are those of Mori[8] and Lambert and Yudkin.[9,10]

Mori's paper was published from the department of Chemical Hygiene in the School of Hygiene and Public Health of Johns Hopkins University so that, presumably, his animals were upon diets approved by McCollum. The composition of the diet employed is not stated, nor are protocols given. Information in regard to diet, age, and weights of animals and duration of the experiments and constancy of results would have greatly enhanced the value of this very short but informing paper. Mori, though he studied the internal organs, regarded the changes in the lacrimal gland as the "most important in the pathological picture of this disease." The change he described was a shrinkage in the cells with other evidences of loss of power of secretion. To the resultant dryness he attributed the xerosis (keratinization) of the cornea and conjunctiva. He also mentions xerotic (keratinizing) changes in the mucous membrane of the larynx and trachea, the principal ducts of the submaxillary, sublingual, and parotid glands and in the ducts of the Meibomian glands. The various salivary glands all showed shrinkage (atrophy) of the cells. The Harderian gland showed changes which can probably be summed up as atrophic. The liver, pancreas, "bowel," kidneys, and thyroid were reported as showing no remarkable change. He made an important conclusion to the effect that "it is necessary to consider the clinical picture of xerophthalmia as a series of secondary symptoms which are indicative of hypofunction of a certain part of the

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[3] Tsuji, K., *Acta scholæ med. univ. imp. Kioto*, 1920-21, iii, 713.

[4] Davis, M., and Outhouse, J., *Am. J. Dis. Child.*, 1921, xxi, 307.

[5] Cramer, W., Drew, A. H., and Mottram, J. C., *Lancet*, 1921, ii, 1202.

[6] Wason, I. M., *J. Am. Med. Assn.*, 1921, lxxvi, 908.

[7] Meyerstein, A., *Arch. path. Anat. u. Physiol.*, 1922, ccxxxix, 350.

[8] Mori, S., *Bull. Johns Hopkins Hosp.*, 1922, xxxiii, 357.

[9] Yudkin, A. M., and Lambert, R. A., *J. Exp. Med.*, 1923, xxxviii, 17.

[10] Lambert, R. A., and Yudkin, A. M., *J. Exp. Med.*, 1923, xxxviii, 25.

secretory apparatus of the organism." It is difficult to account for Mori's failure to pay more attention to and to pursue further the keratinization of epithelium.

Yudkin and Lambert and Lambert and Yudkin in their two papers reported observations made upon white rats which were fed under the direction of Osborne and Mendel. They gave all essential data and a number of protocols of typical experiments. They studied only the eye and the paraocular glands. They could not confirm Mori's conclusion that a drying of the eye and a cornification of the epithelium are the first events in the eye changes. Their studies led them to conclude that the earliest lesions consist of focal inflammatory lesions in the conjunctivæ of the lids and nictitating membrane. The cornification of the cornea they regarded as a secondary phenomenon. "Pathologically, the ocular manifestations of a deficiency of vitamin A are referable to a low grade inflammatory process, originating in the palpebral conjunctiva and spreading to the cornea." Infection and lowered resistance are important factors according to these authors.

In the paper on the paraocular glands, they fail to confirm Mori's finding in the Meibomian glands. They found the most marked changes in the Harderian gland, degenerative changes consisting of swelling, vacuolation, and, occasionally, complete epithelial degeneration. In the lacrimal gland (intraorbital) they found conditions similar to those described by Mori in normal rats as well as in their experimental rats. In the Meibomian gland they could find no appearances not duplicable in normal rats. In this paper they again call attention to inflammatory reactions and occasionally suppuration as a part of the disease picture.

Because of the very meagre total of pathological findings in experimental fat-soluble A deficiency, and because of some conflicting reports, we have decided to state as fully as possible the conditions under which our experiments were made.

The Rats.

The white (albino) rats were obtained from the animal colony of The Wistar Institute of Anatomy and Biology. Two strains, known as the Tyler Strain and the Experimental Colony Strain, were furnished us.

The Diet.

The diet employed was almost identical with that used by Yudkin and Lambert,[9] and designed by Osborne and Mendel.

We used a larger amount of yeast and gave McCollum's[11] salt mixture 185 for inorganic salts.[12]

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[11] McCollum, E. V., Simmonds, N., and Becker, J. E., *J. Biol. Chem.*, 1922, liii, 313.

[12] In a preliminary publication (Wolbach, S. B., and Howe, P. R., *Proc. Soc. Exp. Biol. and Med.*, 1925, xxii, 402) we have incorrectly recorded the diet we employed as regards yeast and inorganic salt mixture.

The diet as employed by us was

Casein	18
Starch	51
Salt mixture	4
Lard	24
Brewer's yeast	3
	100

The casein, starch, lard, and salt mixture were prepared by the Harris Laboratories. The brewer's yeast (trade name Majestic) came from the Liberty Yeast Company, Cambridge, Massachusetts. Distilled water, sufficient to make a dough, was added to the ingredients; small cakes were moulded, each containing five gm. of material, and dried in an oven. The final temperature was sufficient to melt the lard.

The formula for the salt mixture is

NaCl	0.173
$MgSO_4$ anhydrous	0.266
$NaH_2PO_4 + H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2 + H_2O$	0.540
Ferric lactate	0.118
Calcium lactate ($5H_2O$)	1.300

This mixture of inorganic salts was found by McCollum, Simmonds, and Becker to be adequate in the prevention and cure of a form of ophthalmia described by them, and which develops in rats supplied with fat-soluble A.

It is interesting to note that Mori [13] regards this form of ophthalmia as identical with that produced by deprivation of fat-soluble A, but his very brief description of the pathology does not support this conclusion.

The daily ration per rat was 5 gm.

The suitability of the experimental diet we used is shown by the facts that none of the rats studied showed gross or histological evidence of rachitic or scorbutic changes, that young rats continued to increase in weight for 2 to 3 months, that the pathological effects were constant, and finally by the completely normal growth and fertility of rats fed upon the control diet in which the only difference was the substitution of butter fat for lard. The control diet was tested for its curative properties and its administration was always followed by prompt amelioration and disappearance of the signs and symptoms of the deficient state.

Controls.

Control rats on the same diet, but with butter fat (prepared from fresh butter by melting and washing in hot water) in place of lard, grew normally and have been maintained in normal healthy condition for a year.

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[13] Mori, S., *Am. J. Hyg.*, 1923, iii, 99.

The following experiment shows also that rats were fertile and able to produce healthy offspring while upon this control diet.

The rats were from The Wistar Institute, Tyler Strain, and all four were born on Feb. 28, 1924. They were received on Apr. 9, 1924, and kept on a mixed diet of various grains, white bread, and greens until Aug. 22, 1924, when they were placed upon the control diet. On Apr. 14, 1925, that is after 235 days on control diet, Female 93 and Male 94, and Female 95 and Male 96 were placed together.

On May 12, 1925, Female 95 gave birth to six young, five females and one male. The control diet ration was increased to 15 gm. and supplemented by milk, 15 cc. daily. All six offspring were raised to maturity.

On July 4, 1925, Female 93 gave birth to two normal young which she killed, one on July 6, the other on July 10. This female was kept on a 5 gm. ration of the control diet. We neglected to make certain that lactation was possible.

Clinical Course.

The effects of diet deficient in fat-soluble A upon standard bred albino rats as affecting the growth, nutrition, and gross changes occurring in the eye have been described so often and so well that a repetition here is not desirable. Our observations confirm the established order of events, though in no instance did we observe ulceration of the cornea or hypopyon. Loss of power to smell was a late but constant symptom. In male rats, priapism was almost constantly present for 1 to several weeks before death.

Each experimental animal was kept in a separate cage. Excelsior was used for bedding and the cages and vessels for water were kept scrupulously clean. Each animal was fed and watched and after loss of smell occurred, the food was actually placed in its mouth until the ration was consumed.[14]

In common with other investigators we have observed that a much longer period of deficient diet is required to produce symptoms in full grown adult rats than with young, partially grown rats. Similarly, the histological changes occurred more promptly and more extensively in immature than in full grown rats.

The following table of rats which died in direct consequence of the diet illustrates the effect of age and weight.

[14] The success of this investigation is in large measure due to the meticulous care used in the preparation of the diets and feeding of the animals by M. Otto Heinzer.

No. of rat.	Initial weight.	Duration.	Histological changes.
	gm.	*days*	
224	47	63	+++
227	52	61	++
219	56	78	++
216	73	81	
220	75	91	++++
215	81	80	
206	84	77	++
200	86	81	
217	90	78	+
214	91	75	
221	91	109	
222	92	97	++++
211	94	77	++
206	101	89	+++
213	106	109	
204	116	100	+++
203	134	98	+++

The ages of these rats were between 45 and 60 days. Full grown adult rats, aged 150 to 260 days, survived the diet for periods of 150 to 186 days, and on the whole showed less striking histological changes than the younger rats which survived shorter periods. One rat, aged 139 days at the beginning of the experiment and killed after 157 days of deficient diet, showed no keratinization in any organ.

Gross Pathology.

The external appearances of white rats in advanced fat-soluble A avitaminosis have been well described and illustrated. The humped posture, rough coat, emaciation, and encrusted eyelids are familiar signs.

Careful post mortems on thirty-seven rats have revealed few distinctive lesions besides the effects of retardation of growth, the emaciation and the atrophy of certain organs.

We did not record weights and our statements in regard to atrophy or reduction of size of organs are based upon visual comparison only and in the case of small organs by comparing the entire cross-sections as prepared for microscopical study with the normal for corresponding age and weight.

Gross changes which probably are not peculiar to vitamin A deficiency are the

disappearance of fat in adipose tissue throughout the body, diminution of size of liver and spleen, and actual atrophy of the following glands; submaxillary, parotid, lacrimal, Harderian, extraorbital, pancreas, thyroid, pituitary, thymus, and testes The kidneys, parathyroid, and adrenals show no decided decrease in size. The liver becomes small because of absence of stored fat and glycogen, the spleen because of depletion of lymphoid cells. The organs described as atrophic show changes in individual cells when studied microscopically. The Harderian gland becomes paler than normal, the extraorbital deeper colored.

The degree of atrophy varies with different organs. In the case of the submaxillary gland and the Harderian gland, we have occasionally found almost complete disappearance of secreting tissue. Enlarged lymph nodes may be mistaken for submaxillary glands. The atrophy of the testes, parotid, extraorbital gland, and the anterior lobe of the pituitary may be greater than 50 per cent, we have estimated. The thymus gland practically disappears. Marked edema of the submaxillary gland, and, in males, of the testes, takes place during the progress of the atrophic changes.

The distinctive or peculiar gross changes are those due to mechanical factors sequential to the formation and retention of desquamated keratinized epithelial cells. Thus cysts are produced in the submaxillary gland and accessory salivary glands in the base of the tongue and were often found to reach a diameter of 5 mm. Interference with swallowing, because of the large size of the tongue cysts, has been a cause of death in some rats. In the lungs, bronchiectatic cavities were common and occasionally reached a size of 6 mm. in diameter and were so numerous as to be the cause of death. In the urinary tract, great dilatation of pelves and ureters was not infrequent, and complete blockage with desquamated cells was regarded as the cause of death in several rats. The bladder, even more frequently, was found partially filled with desquamated cells.

In male rats, small cysts of similar origin were common in the prostate glands, while the process in the seminal vesicles led to marked contraction and an opaque yellow coloration, almost constant findings in advanced conditions.

The only gross change in the skeleton that we observed was a dorsoventral bowing of the lower cervical and upper thoracic spine, with ventral convexity. Posture, plus athrepsia, we believe accounts for this bowing as the microscopical study after curative treatment as well as of the untreated showed no evidence of rachitic or scorbutic processes in the vertebræ or elsewhere. Individual vertebræ, however, did show alteration in shape in conformity with the spinal curvature.

Histopathology.

Technique.—Zenker's fixative was employed for routine purposes. The routine stain was a modification of Giemsa's,[15] and was used because we believe it has decided advantages in the study of cell changes and is in no respect inferior to the

[15] Wolbach, S. B., *J. Med. Research*, 1919–20, xli, 1.

eosin-methylene blue method. The bone marrow was studied in sternum and ribs, which were fully decalcified in Zenker's fluid. These bones and all soft tissues were embedded in paraffine. The skull, teeth and nares, and large bones were decalcified in 5 per cent nitric acid, embedded in celloidin, and stained with hematoxylin and eosin.

Mitochondria studies were made upon tissues fixed in Regaud's fluid and mordanted further in 3 per cent potassium bichromate. The sections were stained in Altmann's aniline oil, acid-fuchsin, and counterstained with picric acid.

General Account of the Histopathology.

As we have pointed out in a preliminary report,[16] the specific tissue change due to deprivation of fat-soluble vitamin A is replacement of various epithelia by stratified squamous keratinizing epithelium. Atrophy of glands, including ductless glands and the failure to store fat are other striking phenomena demonstrable histologically.

The formation of keratinizing epithelium takes place in the following locations.

1. *Respiratory Tract.*—In the nares including turbinate bones, nasal septum; in the sinuses communicating with the nares; in the larynx, trachea, and bronchi.

2. *Alimentary Tract.*—In the submaxillary glands, the parotid gland, the accessory salivary glands of the tongue and pharynx, and in advanced conditions only in the pancreatic ducts. The stomach, liver, and small and large intestines are not involved.

3. *Genitourinary Tract.*—In the bladder, ureter, and pelvis of the kidney; in the uterus and oviducts, in the epididymis, prostate, seminal vesicles, and coagulating glands. The kidney epithelium and the epithelium of the seminiferous tubules do not keratinize.

4. *Eyes and Glands.*—In the conjunctiva, Meibomian gland ducts, in the cornea, lacrimal gland, Harderian gland, and the extraorbital (lacrimal) gland.

5. *Ductless Glands.*—In the thymus where occasionally we have found greatly enlarged Hassall corpuscles, veritable small cysts filled with desquamated keratinized epithelium.

In all of the glands, alimentary tract, genital tract and paraocular, the appearance of keratinizing epithelium is preceded by atrophy

[16] Wolbach, S. B., and Howe, P. R., *Proc. Soc. Exp. Biol. and Med.*, 1925, xxii, 402.

of the original epithelium and occurs first in the ducts and later in the gland acini. Infection and suppuration are very common, but not invariable and have nothing to do in initiating the epithelial change. The transformation of epithelium in gland acini is seen easiest in the Harderian gland in very late stages of the avitaminosis.

The sequences of the process have been easiest to follow in the respiratory mucosa upon the turbinate bones, in the trachea and bronchi and in the epithelium of the ducts of the submaxillary gland. In all of these locations the process begins in numerous foci and each focus spreads. The rate of growth is rapid as attested by numerous mitoses of the basal cells. The adjacent viable columnar epithelium becomes undermined by the new stratified epithelium and completely separated from its original mesenchymal support before it has lost the appearances and staining reactions of viability. We emphasize the multiple origin of keratinizing foci. (Figs. 11—17.) The foci do not always begin in ducts or structures nearest the outlet where duct or gland epithelium comes in contact with stratified epithelium. Thus keratinization was often found in bronchi in the absence of the change in the trachea, while careful study of the nares has shown no evidence that the epidermis invades. In the uterus, the earliest changes toward keratinization were seen in the uterine glands and not upon the surface epithelium.

Details affecting different organs will be given in the descriptions of the individual organs. A few general considerations are, however, appropriately considered first.

Infection as the immediate cause of the eye changes is generally held at present, on the basis that resistance or natural immunity is impaired by the avitaminosis. We have made no direct attempts to study resistance in infection in this condition. Our own experiences in the care of the rats are in complete opposition to the importance of infection, either as an initiating factor in the pathology or as a cause of death. Our microscopical studies, while giving frequent evidence of bacterial invasion of the nares, the salivary gland, conjunctiva, bladder, and renal pelvis show quite the contrary in other locations.

Advanced keratinization of conjunctiva, cornea, and Harderian gland usually occurred without microscopical evidence of infection. The same is true of the bronchi and bladder and renal pelvis. In

spite of extreme degrees of bronchiectasis, pneumonia was very rare in our rats. We had not a single example of ulceration of the cornea or of hypopyon, though occasional instances of pyogenic reactions in conjunctiva and cornea. In spite of evidence of bacterial invasion, in some instances of the renal pelvis, following keratinization, there was no instance of bacterial invasion of the substance of the kidney.

The disappearance of stored fat in the face of a liberal amount of lard in the diet plainly indicates focussing chemical and physiological studies upon the fat metabolism. Clinical signs and microscopical evidence of the deficiency do not occur until the bulk of stored fat has disappeared. Yet, we have found extensive keratinization in rats with undiminished fat in bone marrow and the presence of occasional plump fat cells in small numbers in many locations, subcutaneous, perirenal, mediastinal, and omental. We have not devoted special study to the so called glandular adipose tissue.[17]

Though familiar with the appearances and locations of this lobular fat tissue we are not convinced of its having special chemical functions and we believe that adequate study of its presence in relation to age and nutrition has not been made. However, some "glandular" fat may still be present in microscopic amounts in well established pathological conditions of fat-soluble A avitaminosis. The presence of minute amounts of stored fat does not, however, exclude the possibility that the missing factor responsible for the cell changes is one derived from fat.

Mucus is another substance which may be followed microscopically, and inasmuch as the commonest seats of the keratinizing changes are mucus-secreting epitheliums, we have tried to establish some association between the two. This has not been possible, the formation of mucus continues in epithelium in which many foci of keratinization are established, while in the intestinal tract secretion of mucus in typical goblet cells appears to be unaffected up to the death of the animal from avitaminosis.

Mitochondria Studies.

All of the organs susceptible to the changes described as well as liver and kidneys of one control rat and two experimental rats were compared for mitochondria content.

[17] Cramer, W., *Brit. J. Exp. Path.*, 1920, i, 184.

One of the experimental rats, No. 100, was an adult 175 days old at the beginning of the experiment. This rat was killed after 181 days on the deficient diet and showed only early lesions of the deficiency, very slight atrophy of glands and small foci of keratinization in the submaxillary gland, the renal pelvis, and epithelium of the turbinates. Plump fat cells were present in abundance in retroperitoneal tissues, poles of testes, and subcutaneous tissue.

The other rat, No. 205, weighed 104 gm., initial age between 45 and 60 days, was killed after 89 days and showed extensive keratinization in turbinates, posterior lingual, submaxillary glands, and moderate keratinization in Meibomian gland ducts, the Harderian glands, bronchi, bladder, and prostate. This rat showed almost complete loss of stored fat, though a few plump fat cells were found microscopically in the polar fat of testes and subcutaneous tissues and many in the bone marrow.

In the liver, pancreas, kidneys, and adrenals all three rats showed similar mitochondria pictures. In some instances the mitochondria were more numerous in the experimental rats as in the pancreas and kidney of Rat 205, and liver of Rat 100 than in the control, due probably to vagaries of technique.

In thyroid, larynx, trachea, bronchi, Harderian glands, extraorbital, lacrimal, submaxillary glands, and intestinal mucosa there was little if any difference between the three rats.

In the submaxillary gland of Rat 205, where the cell atrophy was most marked, the mitochondria were grouped in close apposition to the nuclei and seemed to be reduced in numbers. However, in the keratinizing epithelium in ducts mitochondria were abundant in the basal layer of cells. In a primary bronchus of Rat 100 where only small islands of keratinizing epithelium were established, and in the larynx of Rat 205, mitochondria were found in normal numbers in the ciliated epithelium in contact with the keratinized patches, and also in the basal cells of the latter.

These observations, while few, seem conclusive and indicate that the mitochondrial apparatus is not primarily affected in fat-soluble A deficiency.

Histological Sequences.

In order to avoid constant repetition of objective findings, we shall present much of our microscopical studies in narrative form, basing our account on the study of tissues from rats in succession of progress of the fat-soluble avitaminosis.

The order in which keratinization involves different organs gives no clue to the possible chemistry involved.

In twenty-five rats showing keratinization, studied histologically, the epithelium of the turbinate bones and the submaxillary gland were

always involved. As a rule, keratinization of trachea and bronchi preceded that of the urinary tract, but in four instances the reverse was true. The pancreas was the last organ to show keratinization. The Harderian glands, and the intra- and extraorbital lacrimal glands were affected much later and in lesser degree than salivary glands, respiratory tract, urinary tract, and uterus in the female. In the submaxillary glands, the ducts of purely mucus-secreting alveoli, mixed types, and pure serous types are simultaneously affected by the sequence leading to keratinization.

The atrophy preceding keratinization may be so slight as to require comparison with normal controls for detection. The diminution in size affects the cytoplasm. The nuclei remain unaltered in size. The original cells of an epithelium do not change their characteristics. The cells destined to produce the foci of stratified keratinizing epithelium may be recognized as clumps of two or more deeply basic staining cells beneath the original epithelium. (Fig. 11.) These cells appear at first to form a syncytial mass. Their nuclei are richer in chromatin than those of the original epithelium and contain conspicuous chromatin "knots." Mitotic figures are numerous and growth may take the form of a thin layer of cells underlying the original epithelium or of a circumscribed clump.

In the trachea, these newly formed cells have been found in a layer of single cells completely encircling the lumen underneath the original and still apparently normal epithelium. With growth which is rapid, as attested to by the number of mitotic figures, the cells form an orderly stratified layer applied in normal fashion to the tunica propria; the superficial cells exhibit the sequences of normal keratinization as regards presence of keratohyaline globules and staining reactions. Throughout the duration of life, multiplication of the basal layer of cells continues at a rapid rate, and large numbers of keratinized cells are thrown off. The original epithelium is apparently able to survive for a considerable period upon the surface of the keratinizing epithelium and the microscopic pictures indicate that it is often cast off in sheets. Where the initial growth is in the form of a clump of cells, and this is the more common finding, Figs. 11, 12, 14, and 15, the focus grows by peripheral extension beneath the adjacent and often apparently normal epithelium. Fig. 16. Thus, in the nares and trachea,

mucus-secreting ciliated epithelium with normal staining reactions and normal appearing nuclei may lie upon a keratinizing layer of cells.

The most striking examples of rapidly continued proliferation of the keratinizing epithelium have been found in the bladder, ureters, and renal pelvis and in these locations we have found responses on the part of the supporting tissues suggestive of the behavior of the stroma in epithelial new growths. The orderly apposition of epithelium and tunica propria has been lost, epithelial downgrowths are present and the greatly thickened layer of epithelium has become vascularized by the incorporation of blood vessels of capillary and precapillary size.

We are endeavoring to learn the consequences of "pyramiding" this effect in rats by alternating the deficient diet with the control diet. These observations on the sequences, particularly the focal character of the initial changes, suggests an unequal rationing of cells with the resources remaining in advancing avitaminosis. It is also evident that factors essential to epithelial proliferation are abundantly present in advanced fat-soluble A avitaminosis. Function, possibly to be expressed as maintenance of differentiation is interfered with.

The Integument Muscle and the Supporting Tissues.—Observations have been made only on the skin of the eyelid and base of the ear where the skin was included incidentally in the preservation of the eye and parotid and extraorbital glands. In these locations no striking changes were found. In the most advanced cases of the avitaminosis, slight but demonstrable atrophy of the hair follicles and sebaceous glands was found. The epidermis in a few instances was thinner than in controls and the keratinized layer less pronounced.

The connective tissue throughout the body is unchanged with the exception of three locations where edema was found in the interstitial connective tissue of testes, submaxillary gland, and lungs. This edema in testes and submaxillary gland reaches a height before the atrophy becomes extreme. Exact time relationship has not been worked out, but the edema apparently occurs during the period of early rapid atrophy which, we presume, follows the exhaustion of stored vitamin, and may disappear before death.

In the case of the mucous type of submaxillary gland, the edema liquid stains bluish, without doubt due to admixture of mucus derived from disintegrated cells. Mori has observed this edema with admixture of mucus, and offered substantially the above explanation.

The inference that this edema is the consequence of inability of

epithelium to utilize the materials brought to it by the blood is a natural one but implies functions on the part of the vascular endothelium, as yet not recognized. In the lungs, the connective tissue of the pleura, septa, and perivascular and peribronchial supports is the seat of moderate degrees of edema, never recognized in gross, and as yet not to be correlated with other findings in the body.

Skeletal muscle was studied in preparations of the skull, tongue, thorax, scapula, spine, and long bones. No change was found attributable to the avitaminosis. Waxy degeneration was found in a few cases, and only in association with infection. Bone and cartilage were studied routinely in costochondral junctions of ribs, in the sternum, the skull, and in a few extreme cases of the avitaminosis in vertebral and long bones. No specific change was found. Evidence of complete cessation of growth was the rule in advanced cases, but there was no evidence of osteomalacia, rachitis, or scorbutus.

Hess, McCann, and Pappenheimer[18] failed to produce gross skeletal changes in rats on a diet deficient in vitamin A. They have described the microscopic appearances due to lack of bone growth and we cannot add to their account.

The Teeth.—Routine sections of the skull through the nares at three levels were made from twenty-two rats, thus affording opportunity to study the incisor teeth of the upper jaw. Gross changes in the teeth were not noted in life except that the rate of growth was much slower than in rats upon other diets, water-soluble B deficiency and high protein diets, for instance. Actual wearing down of the incisors in a few rats indicates that complete cessation of growth may occur. Microscopical changes, usually of minor degree were found regularly in the odontoblast layer and frequently in the pulp. Our series affords a complete sequence from the earliest demonstrable shrinkage of the odontoblasts to complete disappearance. Preceding complete disappearance, irregular downgrowths of odontoblasts occur and these produce irregular deposits of dentine. Odontoblasts, while undergoing atrophy continue to produce dentine and presumably, due to the separation of the individual cells, become incorporated in the dentine matrix. Another less frequent finding was the presence of islands of osteoid tissue, incorporating and surrounded by osteoblasts. A careful study of early stages of this lesion has convinced us that the connective tissue cells of the pulp take up the function and morphology of osteoblasts because the first step in the process is the deposit of a hyaline homogeneous matrix between normal appearing

[18] Hess, A. F., McCann, G. F., and Pappenheimer, A. M., *J. Biol. Chem.*, 1921, xlvii, 395.

connective tissue cells. While superficially these changes resemble those in latent scorbutus in guinea pigs,[19] the fundamental phenomenon is probably different, as we have shown[20] that in scorbutus inability to produce intercellular matrices is the first phenomenon and not cell atrophy. Again, as in the consideration of edema of the testes, we may make the query, do these deposits of osteoid-tissue in the pulp represent the utilization of transported material designed for odontoblasts. Enamel formation ceases. The enamel-forming cells in advanced stages are either shrunken and atrophic or replaced by a narrow layer of stratified, non-keratinizing epithelium.

The Respiratory Tract.—The replacement of the respiratory mucosa of the nares, (Figs. 1 and 2) accessory sinuses, larynx, trachea (Figs. 3 and 4) and bronchi (Fig. 17) by stratified squamous keratinizing epithelium has been mentioned and the histological sequences described. In the nares the olfactory glands (Bowman's glands) also become replaced by keratinizing epithelium. A purulent exudate in the nares is common, but not constant. In numerous instances there was extensive replacement of the respiratory mucosa by keratinizing epithelium without the presence of an exudate. Again, an extensive puriform infiltration and exudation was present in rats killed before keratinization had begun. From our material we are able to conclude that infection of the nares is common in this avitaminosis, but that the specific epithelial changes occur wholly independently of infection.

In the larynx, trachea, bronchi, and their glands, evidences of infection may or may not be present, with or without replacement of normal mucosa by keratinizing epithelium. The lungs show the pathology of secondary changes, chiefly due to occlusion of bronchi with desquamated keratinized cells, *i.e.* bronchiectases which may or may not show evidences of infection. The occasional minor degrees of bronchitis and bronchopneumonia which we found are without distinctive pathology.

The olfactory epithelium and the sensory epithelium of Jacobson's organ were always intact except for instances of inflammatory changes and invasion by growth of adjacent keratinizing epithelium.

The Alimentary Tract.—In the esophagus, stomach, and intestines no changes were found except in rare instances of a slight degree of atrophy, perceptible only by comparison with controls of the glands (Brunner's) of the duodenum.

The pancreas in advanced cases showed moderate atrophy of the secreting cells. The islands of Langerhans were consistently normal in the entire series. In the main branches of the pancreatic duct in a few rats there were foci of early formation of keratinizing cells.

The liver showed no lesions peculiar to the avitaminosis.

The Submaxillary Gland. Fig. 5.—The earliest changes to be noted, but only by careful comparison with controls, in the series, is slight atrophy of the gland acini, both mucous and serous types, and atrophy of the duct epithelium. Islands

[19] Höjer, J. A., *Acta pædiat.*, 1924, iii, suppl.

[20] Wolbach, S. B., and Howe, P. R., *Proc. Soc. Exp. Biol. and Med.*, 1925, xxii, 400.

of keratinizing cells appear in the complete absence of evidences of infection or any cellular infiltration whatever; and before apparent atrophy of the gland itself has occurred. Edema of the submaxillary gland occurs apparently in the most active period of atrophy and like that of the testes is transient—disappearing before maximum atrophy. The edema is strictly localized and is confined by the capsule of the gland. The liquid in mucus-secreting lobules is basic staining and study has shown that this property is due to admixture of mucus from gland cells which disintegrate. We are inclined to attribute the cell disintegration to the effects of the edema, sequences in the tunica propria can be followed from hyaline swelling and fragmentation to disappearance. Cells separate from their acini and become free in the liquid. The nuclei of the epithelial cells degenerate, the cells become rounded and finely vacuolated. Other cells present are lymphoid cells, small numbers of polymorphonuclear leucocytes, spindle cells, presumably from the supporting tissue of the gland, and rarely eosinophilic leucocytes and red blood corpuscles. Capillaries and larger blood vessles may traverse edematous spaces, but hemorrhages do not occur and the disposition of the blood vessels as edema and atrophy progress has not been determined.

As the avitaminosis progresses, the keratinization of ducts and atrophy of gland tissue proceed, in most instances complicated in later stages by infection. Without infection we find an infiltration of the gland stroma with lymphoid and plasma cells. The gland cells become vacuolated and occasionally invaded by polymorphonuclear leucocytes. The connective tissue of the stroma increases. In mucus-secreting alveoli there is a noticeable increase of small, deeply staining, non-vacuolated epithelial cells which persist after desquamation and disappearance of the original cells. The same process occurs later in the serous alveoli, and in the most severe grades of the deficiency these replacing cells become squamous and keratinizing. Eventually the gland as a whole becomes markedly fibrosed and filled with cysts lined with keratinizing epithelium. Infection complicates the picture as it causes complete destruction of the epithelium lining cysts and repair by granulation tissue accompanied by foreign body reactions to the retained keratinized cells. As the cysts, derived from ducts, dermoid in character, reach dimensions of 5 to 6 mm. pressure atrophy effects ensue in adjacent gland tissue.

The accessory salivary glands of the base of the tongue and the pharynx show the same sequences as those of the submaxillary gland and in these locations the gamut of events may take place independently of infection. (Fig. 6.)

The parotid glands rarely showed evidence of infection. Keratinization of ducts and atrophy of the gland occurred much later in the progress of the deficiency than in the submaxillary gland, and but few instances of cyst formation, due to accumulation of keratinized cells occurred in the series. The sequences, though occurring late, are the same as in the submaxillary gland.

The Urinary Tract.—In the epithelium of the bladder, Fig. 10, ureters, and pelvis of the kidney (Fig. 8) the original epithelium becomes replaced by keratinizing epithelium which develops as in other locations from underlying nests of cells. In

the kidneys, the apices of the pyramids become covered by a thick layer of keratinizing epithelium, while the epithelium of the pelves, ureters, and bladder show the most remarkable pictures encountered in this study. In these latter locations there is evidence of very rapid growth of the epithelium; in some instances keratinization ceased. Mitotic figures are to be found in every field of a 3 mm. immersion objective and frequently there are two to four mitoses per field. In the bladder invaginations and dermoid cyst-like formation occur. In ureter, pelves, and bladder epithelial downgrowth resulting in the incorporation of blood vessels is frequent. The behaviors indicate growth power suggestive of neoplastic potentiality.

The Male Genital Tract.—Edema of the testes during atrophy of the seminiferous tubules reaches extreme degrees. The liquid lies outside the basement membrane, the connective tissue between the tubules does not seem to be permeated by liquid, so that the appearance in sections is that of tubules floating in a liquid medium. The liquid is evidently rich in protein material as it furnishes a heavy precipitate of coarse hyaline globules. The atrophy of the tubules when complete leaves apparently only cells derived from the sustentacular cells. The cells frequently have two to four nuclei. Early stages in the atrophy show spermatids with scattered chromatin particles and a structureless layer of material, probably derived from the cytoplasm of these cells.

In the epididymis a more diffuse interstitial edema occurs. The cells of the ductus epididymidis become slightly atrophic but retain their normal characteristics. The cells of the efferent ductules of the testis apparently increase in number in early stages of the avitaminosis inasmuch as they form a deep epithelium layer, often with large vacuoles between cells and occasionally with secondary gland-like formation of cells with cuticular borders bounding a lumen. Subsequently shrinkage of cells takes place and rarely islands of squamous keratinizing cells make their appearance in the basal layer of cells. Fig. 15. In one instance the ducts of the epididymis and testis were filled with pus.

The prostate gland, Fig. 9, seminal vesicles, and coagulating gland exhibit with regularity the sequence of atrophy, keratinization, and fibrosis, and the prostate frequently becomes the seat of a puriform exudation.

The Female Genital Tract.—The mucosa of the uterus, and later that of the oviducts, becomes replaced by stratified, keratinizing epithelium. The process originates in numerous foci as in other organs and begins in the uterine glands as well as in the surface mucosa.

No characteristic pathology has been found in the ovaries. We have not made sufficiently careful comparisons with normal ovaries to be certain that some degree of retardation of follicle formation does not take place, but even in advanced stages of the avitaminosis, ova and follicles in all stages of development up to maturity are found. Mitotic figures are present in the follicular cells, and apparently ovulation occurs as corpora lutea are found in various stages of formation and regression.

The Circulatory System.—We have found no change in blood vessels or in the ability to form new capillaries in repair by granulation.

Focal lesions in the myocardium were found with considerable regularity in sixteen rats out of nineteen, which showed keratinizing epithelial changes. Control rats and three adult experimental rats kept on the deficient diet for periods of 50 to 120 days, but which did not develop the characteristic epithelial changes of the deficiency, did not show lesions of the myocardium. The lesions are focal, usually not numerous, and represent the sequences of degeneration of a few contiguous muscle fibres—acidophilic staining, loss of striations, vacuolization, and fragmentation, and the consequent reparative processes, terminating in cicatrization. Correlations of these myocardial lesions with epithelial changes show that the more advanced the latter the more numerous the former.

Blood and Lymphoid Tissues.—No study of the morphology of the blood cells was made in life. Bone marrow was studied in sections from ribs and sternum from every autopsy. We could find no characteristic differences between the marrow of normal and control diet rats and the marrow from rats with advanced lesions of vitamin A deficiency. In advanced deficiency, fat completely disappears and the fat cells are represented by large connective tissue-like cells. Slight edema with fibrin threads were seen in a few cases. There is no decrease in numbers of megacaryocytes. Reduction in numbers of cells concerned in hematopoiesis was apparent in a few marrows, but a greater depletion of blood-forming cells was found in the marrows of two rats kept upon a water-soluble B deficient diet (casein 18 gm., butter fat 20 gm., starch 58 gm., McCollum's salt mixture 185, 4 gm.) for 3 and 4 months respectively.

The spleen as the deficiency progresses, becomes more and more depleted of lymphoid and erythrocyte-forming cells. Megacaryocytes completely disappear. Phagocytic cells laden with hemosiderin pigment become more numerous so that the end-result is a spleen with no trace of hematopoiesis or lymphopoiesis, containing as many pigmented cells as lymphoid cells. The secondary follicles have disappeared. The reticulum is not increased. The spleens of normal rats vary in number of hemosiderin-containing cells, and frequently contain many. The spleen of a water-soluble B deficient rat showed a comparable degree of atrophy and pigmentation to many of the fat-soluble A rat spleens. The atrophic changes and pigmentation of the latter are accordingly not characteristic of the A deficiency.

Lymph Nodes.—The lymph nodes in proximity to the submaxillary gland and the bronchial lymph nodes usually were larger than in control rats. The enlargement is due in part to the accumulation in lymph sinuses of cells concerned in the disposal of products from atrophying and occasionally infected tissues, and in part to lymphoid hyperplasia. In mesenteric lymph nodes we found no departure from the normal.

The Ductless Glands: Thymus.—Extreme reduction in size, owing to almost complete loss of small thymic cells is constant in established vitamin A deficiency.

The thymic corpuscles (Hassall's corpuscles) from two rats are greatly enlarged and duplicate the human type. They consist of cyst-like structures lined with flattened epithelium and filled with masses of concentrically arranged hyaline scales, which we believe to be desquamated keratinized cells. In one out of four thymuses from control rats we found very much smaller cavities with hyaline material in Hassall's corpuscles. Our observations are too few to be conclusive, but indicate that the thymic corpuscles are epithelial and respond to the vitamin A deficiency as do other epithelial structures.

The Adrenal Gland.—We found no noteworthy response, gross or histological, to the deficiency.

The thyroid, parathyroid, and anterior portion of the pituitary gland all respond by atrophy. In the thyroid, this is evidenced by reduction in size of the cells and diminution of colloid content of the acini. In the parathyroid, moderate, and in the pituitary, striking reduction in size of the gland cells occurs, but we could find no other change and the architecture of both glands remains normal.

The Nervous System.—As the rats exhibited no symptoms pointing to nervous system lesions, the peripheral nerves were not studied. No lesions were found in brain, cerebellum and sympathetic ganglia. The ganglion cells of the myenteric plexus were normal whenever found.

The Eye and Paraocular Glands.—The eyes, including the globe, the lids, nictitating membrane, the intraorbital lacrimal gland, the Harderian glands, and the extraorbital lacrimal glands from eighteen rats were studied.

We have found no qualitative differences in the behavior of the eye and its glands as compared with other organs. Mori and Lambert and Yudkin have described the important featues of the pathology, but disagree in their interpretation. Lambert and Yudkin give a much greater importance to infection than does Mori who ascribes the keratinization of the cornea to dryness secondary to loss of secretion. Lambert and Yudkin state that the corneal lesions are secondary to the lesions of the conjunctiva of the lids and nictitating membrane. These authors have missed entirely the specific nature of the keratinizing change which they saw only on the cornea, and treat the pathology of the eye as results of infection secondary to loss of immunity caused or favored by atrophy of the paraocular glands.

Our own observations disprove that infection plays an important rôle in producing the clinical picture of the ophthalmia or that it is responsible for initiating the keratinizing change. In only one rat, initial weight 134 gm., which died on the 98th day of the experiment, did we find the histology of an infectious process.

As Lambert and Yudkin state, lacrimation persists after the clinical signs of the ophthalmia are established. This we confirm and histologically have found that advanced keratinization of cornea and conjunctival epithelium may exist without appreciable atrophy of the Harderian, intra-and extraorbital lacrimal glands.

The substitution of a keratinizing epithelium in conjunctiva and cornea is the first change we have found. The mucous cells of the conjunctiva persist after this

change has occurred and are to be seen overlaid with keratinized cells. Subsequently the mucous glands of the conjunctiva atrophy and disappear. Desquamated keratinized cells are in all probability rapidly removed from the conjunctival sac in the first stages of the deficiency. Eventually they accumulate and may adhere to the lid margins where the keratinized cells cohere to a greater extent than elsewhere. In late stages the basal cells of the conjunctival epithelium show slight, irregular downgrowth and there is an increase of capillaries in the underlying connective tissue. At this stage accumulations of mononuclear cells, lymphocytes, and endothelial leucocytes are common. Polymorphonuclear leucocytes invade degenerating mucous cells after these have become completely embedded by the cells of the keratinizing epithelium, but actual proof of infection occurred but once in the series. The epithelium over the nictitating membrane shares in the general process and we have not found it to be a locus of early infection.

The Meibomian glands we find in accordance with Mori do respond, but not strikingly. In a few cases only were the ducts occluded by keratinizing cells so that we attribute but a minor rôle to these glands in producing the picture of the ophthalmia.

The substantia propria of the cornea after keratinization is established, becomes edematous and vascularized by ingrowth of capillaries from the sclera. (Fig. 7.) This response we believe to be due to the greater physiological demands from the more active and changed epithelium. In very late stages of the deficiency wandering cells of all types, including polymorphonuclear leucocytes appear in the cornea. The external basal membrane (Bowman's) not easily demonstrable in the normal guinea pig eye, becomes wholly unrecognizable. The internal basal membrane (Descemet's) remains unchanged. Within the eye we have seen no pathology. The epithelium of the ciliary process undergoes no change.

The Harderian gland responds earlier than the lacrimal glands, later than the submaxillary glands, and undergoes atrophy and keratinization, first in the ducts, later in the acini. The sequences are identical with those described for the submaxillary gland and will not be repeated here. Early evidence of atrophy as pointed out by Lambert and Yudkin is the disappearance of the yellow deposit in the gland lumina as seen after Zenker fixation. Complete disintegration of gland acini and eventual conversion of the gland into a series of cavities lined by stratified epithelium and filled with desquamated keratinized cells is the end-result as observed by us.

The intraorbital and the extraorbital lacrimal glands respond late, first showing atrophy and finally keratinization of the ducts.

Correlation of the eye pathology with that in other organs shows that the eye changes come later than the changes in the respiratory tract and in the submaxillary glands. The epithelium of the eye and its glands respond as do other epithelial structures and in respect to some other organs show less susceptibility to the vitamin A deficiency. The vascularity of the conjunctiva and the innervation of

the cornea and conjunctiva probably contribute largely to the picture during life. The incrustation of the lids is due to adherence of desquamated cells. Infection with destructive lesions of the cornea must be regarded as accidental and need not occur in animals in which the deficiency has been carried to an end-result.

Vitamin A Deficiency in the Human.—There has been but one pathological description of a fatal human case.[21]

This post mortem, done in the pathological service of one of us at the Children's Hospital, is adequately recorded. We have recently restudied the tissues and can add nothing of importance. Keratinization of the epithelium of the renal pelvis, and large size of Hassall's corpuscles in the thymus were overlooked. The keratinization of the pancreatic ducts is more striking than any seen by us in the pancreas of rats. In respect to the keratinizing change in lungs, uterus, submaxillary gland ducts, and thymus, the picture was identical with that in our experimental rats. The order of changes in this human case was somewhat different in that the salivary glands showed only slight changes. The peculiar swollen epithelial cells with nuclear inclusion bodies in the ducts of the parotid and submaxillary glands are unlike any change seen in rats. The lacrimal gland showed only slight changes and no keratinization. The eyes were not removed for study.

The importance of this human case is that it did show the epithelial changes which we have produced in rats and, therefore, contributes irrefutable evidence for an important part of our thesis to the effect that vitamin A deficiency affects epithelial tissues in a specific manner.

SUMMARY AND CONCLUSIONS.

The specific tissue changes which follow the deprivation of fat-soluble vitamin A in albino white rats and in the human concerns epithelial tissues. This effect is the substitution of stratified keratinizing epithelium for normal epithelium in various parts of the respiratory tract, alimentary tract, eyes, and paraocular glands and the genitourinary tract. We have described the morphological sequences which clearly show that the replacement of epithelium arises from focal proliferation of cells arising from the original epithelium

[21] Wilson, J. R., and DuBois, R. O., *Am. J. Dis. Child.*, 1923, xxvi, 431.

and not by differentiation or change of preexisting cells. Young rats respond to the deficiency more promptly than adults.

Growth activity of epithelium is not diminished. On the contrary, there is convincing evidence that it is greatly augmented. In a few of our animals the behavior of the replacing epithelium in respect to numbers of mitotic figures and response on the part of connective tissue and blood vessels suggests the acquisition of neoplastic properties. While the epitheliums which are the seats of these changes are largely of covering types, glandular epithelium is involved, specifically in the paraocular glands and salivary glands. It is highly probable also that the epithelium of gland ducts, respiratory mucosa, and genitourinary tract have secretory functions so that we conclude that the deficiency results in loss of specific (chemical) functions of the epitheliums concerned, while the power of growth becomes augmented. Explanation for the substitution of a chemically inactive (nonsecretory) epithelium, common in type for all locations, remains a matter of speculation.

We can only speculate also in regard to the absence of change in the epithelium of such organ as the liver, parenchyma of the kidney, stomach, and intestines. The significance of the order or sequence in which different organs exhibit this change has not been determined. In general the respiratory mucosa in nares, trachea, and bronchi respond first, then the salivary glands, eye, genitourinary tract, then paraocular glands and pancreas, although as has been noted there are exceptions to this order.

Our studies show that the mitochondrial apparatus is not primarily affected. Study of individual cells indicates that the first morphological evidence of avitaminosis will be found in the nucleus. We have not devoted sufficient study to be certain, but an increase of chromatin and in some instances in size of nucleoli are early morphological manifestations.

Other important effects of fat-soluble A deficiency are atrophy of glandular organs, emaciation, localized edema of testes, submaxillary gland, and connective tissue structures of the lungs and focal myocardial lesions. From our own limited experience with rats fed on a water-soluble B deficient diet and from work by Cramer, Drew, and Mottram,[5] the loss of fat in water-soluble B deficiency is as great, if

not greater than in vitamin A deficiency, so that for the present we assume that this is not a specific manifestation of any one avitaminosis. The same applies to glandular atrophy. Both of these effects probably concern the nutrition as a whole and may be ascribed to inanition.[22]

The occurrence of transient edema in testes and salivary gland coinciding with a period of maximum atrophic change, suggests the hypothesis that this edema is the result of failure of epithelium to utilize transported material, which leads further to the hypothesis that the capillaries of these organs are differentiated in regard to permeability to the respective materials utilized by the cells. It would seem that in the case of the testis we have a unique instance of complete atrophy producible at will without impairment of circulation and supporting tissues. This phenomenon may possibly be followed with advantage in the study of the mechanism of edema.

Vascularization of the cornea, as we have shown it to be independent of infection, must be a physiological response to the increased demands of the rapidly growing epithelium which has replaced the corneal epithelium.

We have assumed throughout this work that the diet on which we kept our animals was deficient in respect to a single substance or group of substances having similar physiological properties, designated by the term fat-soluble vitamin A. Whether or not more than one so called vitamin or accessory substance was missing in the diet we employed does not affect the theoretical importance of the morphological results.

Work by Evans and Bishop[23] would indicate that other factors affecting fertility in addition to the so called antixerophthalmic or vitamin A factor may have been missing.

Our own experience leads us to believe the specific effects we have described upon epithelial tissues were in all probability due to withdrawal of a single factor. We have shown how these effects, that is the replacement of uterine epithelium by keratinizing epithelium can account for sterility in the female. Whether or not the atrophy of the

[22] Jackson, C. M., Inanition and nutrition, Philadelphia, 1925.

[23] Evans, H. M., and Bishop, K. S., *J. Am. Med. Assn.*, 1923, lxxxi, 889.

testis is due to the same factor remains to be proved, but presumptive evidence is strong that this is the case.

The study of the reverse changes that follow in the rapid amelioration when the rats are restored to an adequate diet has been begun and will be reported later.

We have shown that the substitution of keratinizing epithelium in all locations is not secondary to infections, and presumably is a primary effect of the withdrawal of factors essential for the chemical activities or maintenance of differentiation of the epitheliums concerned. It is, of course, possible that the phenomenon is produced in a roundabout way in that it may be secondary to the effects of the avitaminosis upon the metabolism of tissue-sustaining substances. This possibility is supported by the cessation of growth of the skeleton and teeth, although we know that other avitaminoses produce retardation of growth.

EXPLANATION OF PLATES.

PLATE 37.

FIGS. 1 and 2. Turbinate bone at 43 and 93 diameters respectively. To show keratinizing epithelium and accumulation of desquamated cells. Rat 103. Initial age 150 days, died after 180 days on experimental diet.

FIGS. 3 and 4. Trachea at 43 and 257 diameters respectively. Showing complete replacement of respiratory mucosa by stratified keratinizing epithelium. Rat 206, initial weight 101 gm. Died after 89 days on experimental diet.

PLATE 38.

FIG. 5. Salivary gland. 50 diameters. Atrophy; a dilated duct with keratinizing epithelium. Infected. Rat 105. Initial age 54 days, died after 160 days on experimental diet.

FIG. 6. Base of tongue. Keratinizing cysts of accessory salivary gland origin. 50 diameters. Rat 103.

FIG. 7. Cornea. 180 diameters. Vascularization of substantia propria. Keratinization of epithelium. Rat 105. Initial weight 104 gm. Killed on 89th day on experimental diet.

FIG. 8. Kidney. 50 diameters. Keratinizing epithelium on pyramid. Rat 105.

PLATE 39.

FIG. 9. Prostate gland with atrophy, fibrosis, keratinization. 95 diameters. Rat 103.

FIG. 10. Bladder. Rat 103. 95 diameters.

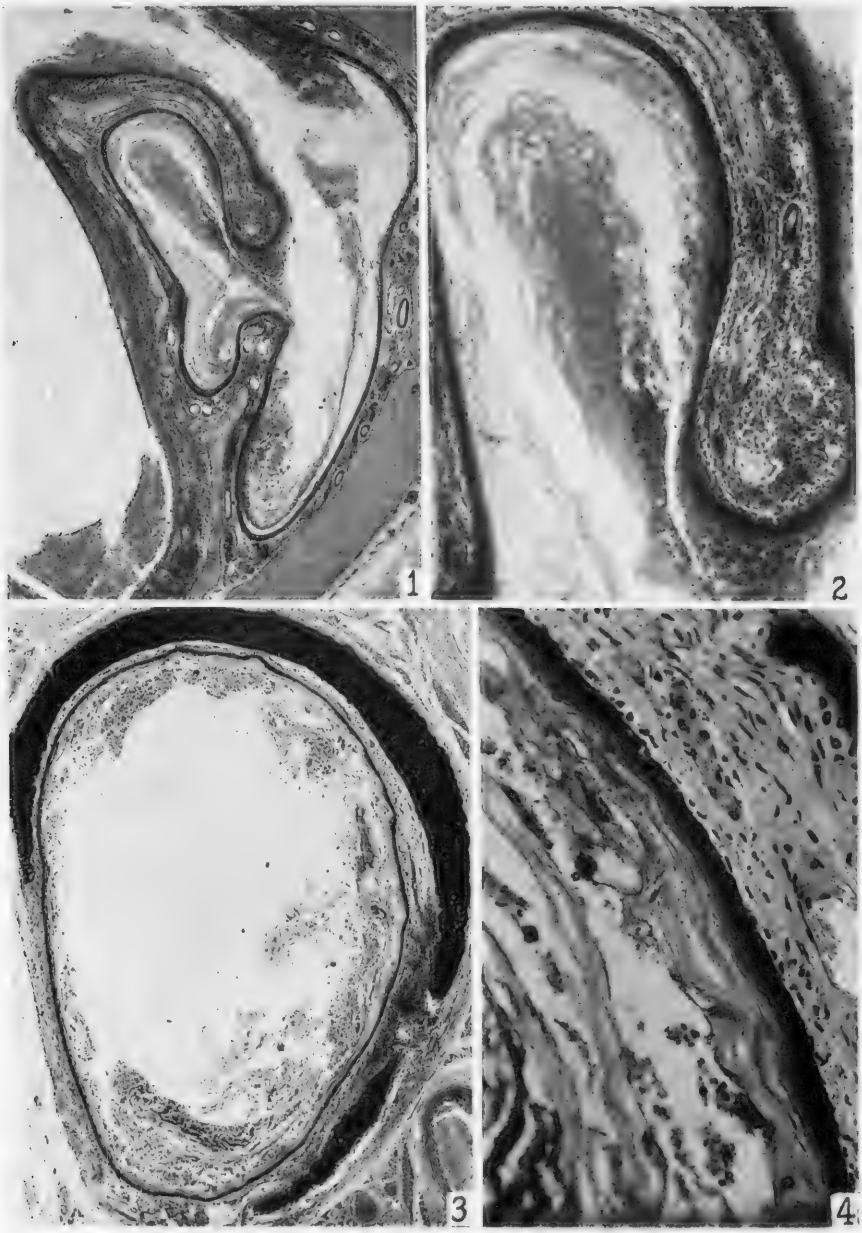
FIGS. 11 and 12. Respiratory mucosa, turbinate bones. 656 diameters. Early foci of keratinization. Rat 100. Initial age 175 days. Killed on 181st day of deficient diet.

FIG. 13. Posterior nares, to show small foci of established keratinization. 173 diameters. Rat 105.

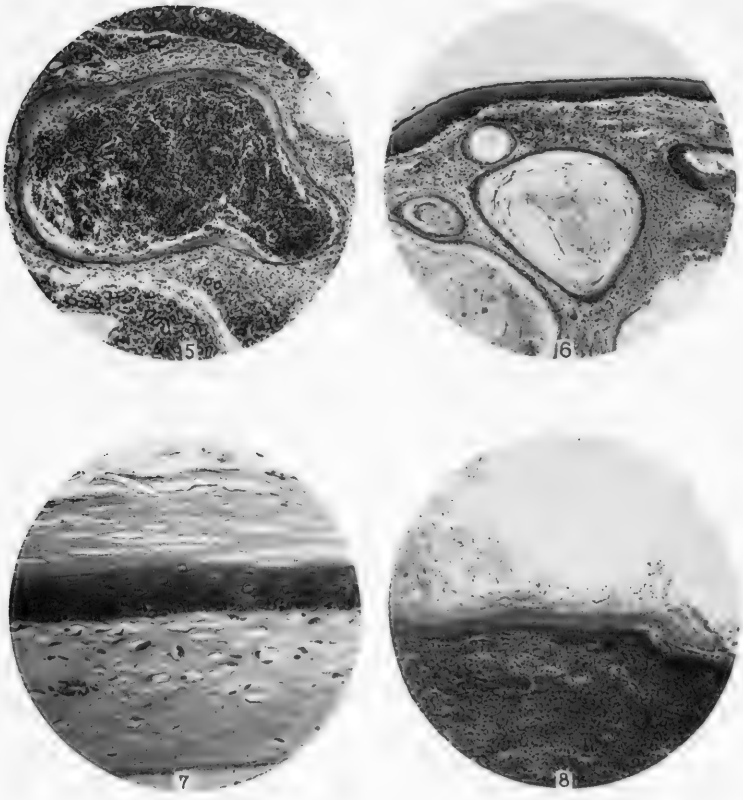
PLATE 40.

FIGS. 14 and 15. From Rat 100. 600 diameters. From trachea and efferent ductule of testis respectively. To show foci of initial change towards keratinization.

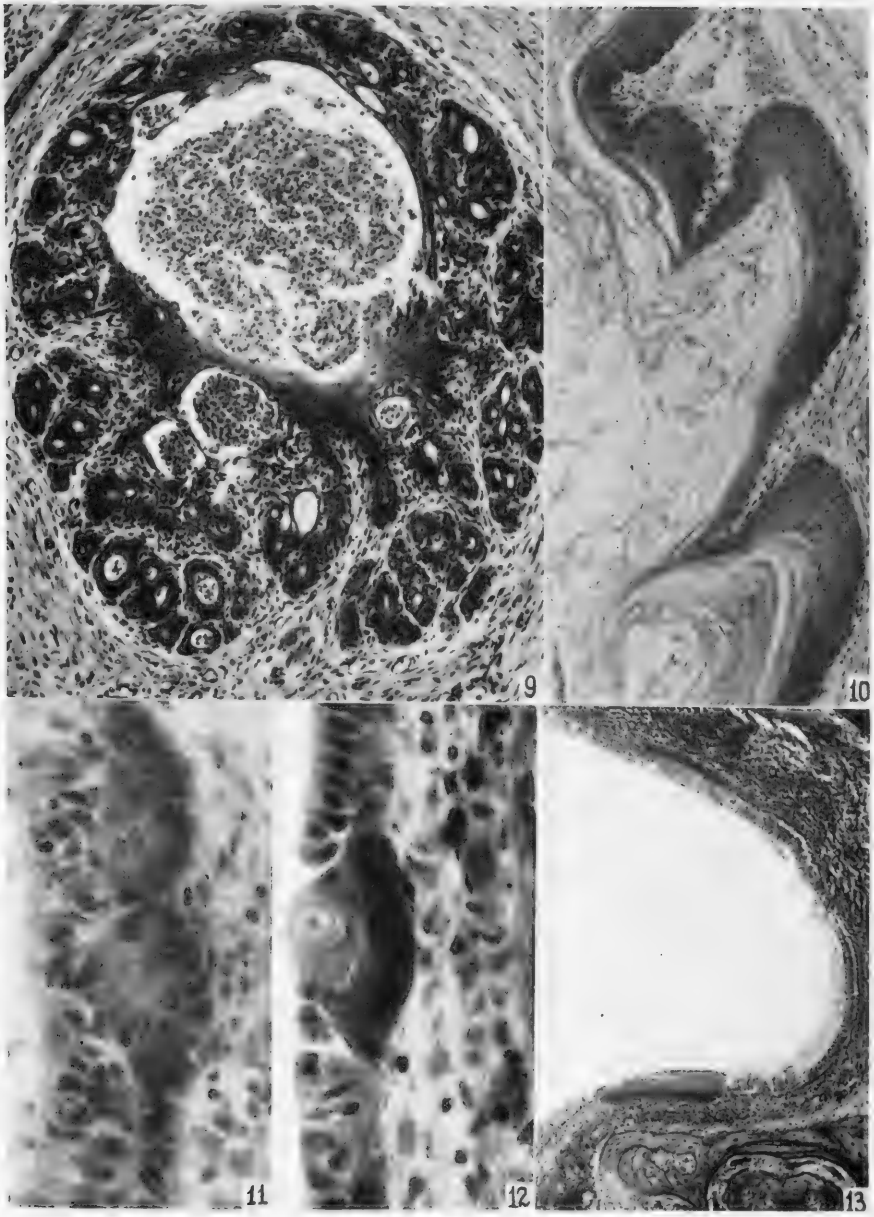
FIGS. 16 and 17. From rat 100. 600 diameters. Fig. 16, from turbinate bone shows small focus of keratinization with extension and undermining of adjacent epithelium. Fig. 17 from a bronchus; a focus of early keratinization.



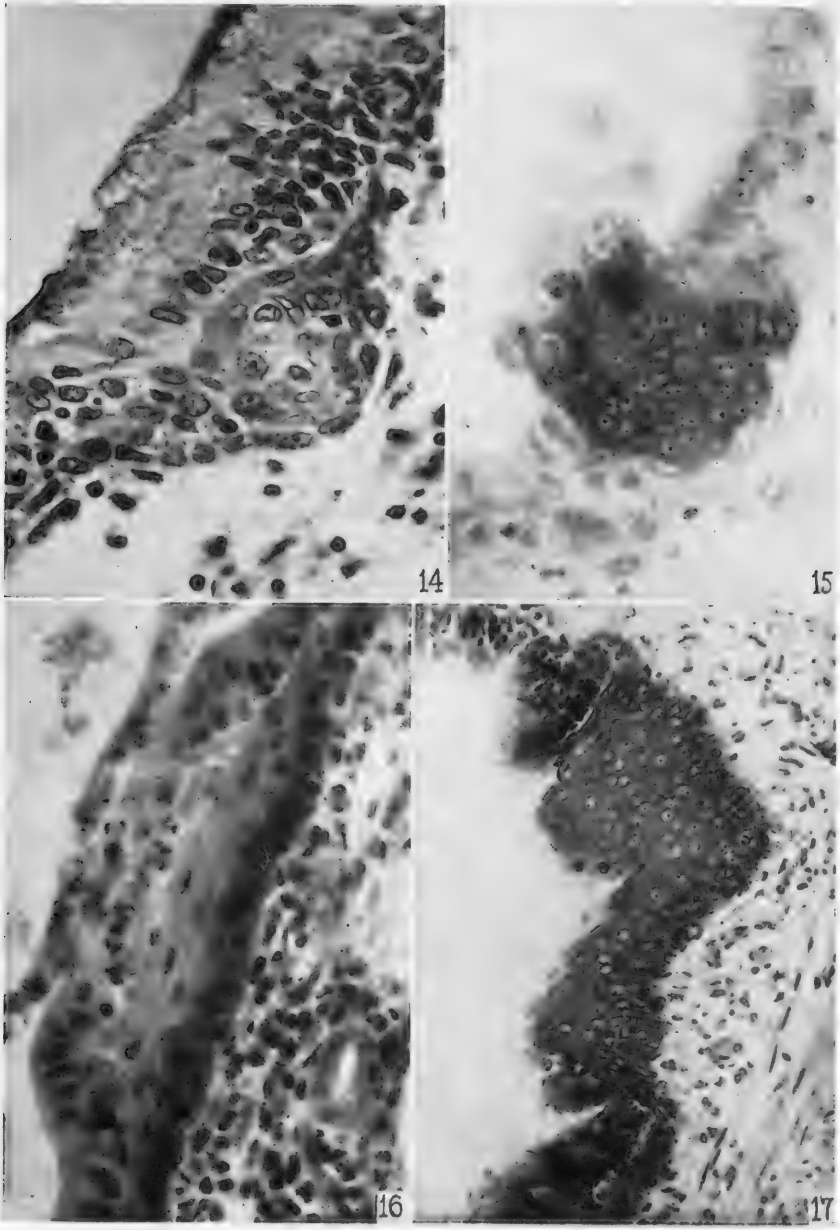
(Wolbach and Howe: Tissue changes after vitamin A deficiency.)



(Wolbach and Howe: Tissue changes after vitamin A deficiency.)



(Wolbach and Howe: Tissue changes after vitamin A deficiency.)



(Wolbach and Howe: Tissue changes after vitamin A deficiency.)

THE ALVEOLAR PORES OF PNEUMONIA.*

By WILLIAM SNOW MILLER, M.D.

(From the Anatomical Laboratory of the University of Wisconsin, Madison.)

Plates 41 and 42.

(Received for publication, September 17, 1925.)

In a previous report (6) some of the factors leading to the formation of alveolar pores were discussed. It was there shown that in the early inflammatory stage of acute lobar pneumonia there is an engorgement of the blood capillaries followed by a serous exudate into the alveoli in which epithelial cells, leucocytes, and red blood corpuscles are present. These elements provide the necessary ferments by which the blood serum is converted into fibrin and the classic exudate of red hepatization is formed.

The walls of the pulmonary alveoli are made up of a network of capillaries, on each side of which there is a network of branching and anastomosing fibrils (the reticulum), reinforced here and there by elastic fibers, an occasional bundle of collagenous fibers, and covered on the outside by a layer of epithelial cells.

The epithelium which covers the walls of the alveoli is frequently described as being of two types; one made up of large non-nucleated plates, the other of small nucleated cells which are often arranged in groups. In other words, the epithelium is said to be discontinuous. This, however, is not the case.

At birth, before respiration is established, the epithelial cells are more or less cuboidal in shape and completely line the walls of the alveoli. With the establishment of respiration and the full distention of the lung, they are stretched out into thin squamous cells and their nuclei are often indistinct; as soon, however, as atelectasis takes place, they return to their non-distended condition and the alveoli are found to be lined with cuboidal cells which, not infrequently, give to the collapsed alveoli the appearance of small bron-

* Aided by a grant from the National Tuberculosis Association.

chioli, from which they can be distinguished by the absence of smooth muscle in their walls.

When from any cause a serous exudate is poured out into the restricted space beneath the epithelium the cells are pushed off by the *vis a tergo*. Occasionally it happens that this is so equally applied that the epithelium lining an alveolus is pushed off as a continuous sheet. In Fig. 1 such is the case, and various degrees in the conversion of squamous cells into nearly detached spheroidal cells can be recognized. In the alveolus in the upper right quadrant of Fig. 1 what appears to be a large non-nucleated plate can be seen closely applied to the alveolar wall. Sections situated immediately above and below the one from which the photomicrograph was taken show the flattened nuclei belonging to the individual cells. The opposite is true of the alveolus in the center of the photomicrograph; sections immediately above and below the one photographed show what appear to be long non-nucleated plates where nuclei appear in Fig. 1 and by careful examination the boundaries of each cell can be made out.

Shedding of the epithelium constitutes but one step in the formation of a pore. If the epithelium on the opposite side of the alveolar wall remains intact, then no pore can result from the shedding of the epithelium on one side of an alveolar wall. The epithelium on the opposite side of a given alveolar wall may be pushed off by the accumulation of serum behind it, but this must take place from diametrically opposite sides of an alveolar wall to play a part in the formation of a pore.

When there is an extensive shedding of the epithelium on one side of an alveolar wall a free exit is provided for the serum through the sieve-like openings in the network of the reticulum. Should this occur on both sides of an alveolar wall, as so frequently happens in the mechanical edema of mitral stenosis associated with insufficiency of the valves, then the alveoli on each side of the alveolar wall are filled with serum in which free epithelial cells can be seen assuming a more or less spheroidal form. It is on account of this extensive shedding of the epithelium, and the absence of fibrin threads, that pores are so difficult of demonstration in a mechanical edema, although they may be as numerous as in a lobar pneumonia.

The clinical history of the case from which the sections used to illustrate this paper were taken, is unknown to me. The lung was one of a number of specimens sent to me. It proved to be from an early case of lobar pneumonia, and from numerous sections three were selected for photomicrographs to illustrate steps in the formation of alveolar pores.

In Fig. 2 there has been an extensive exudate poured out from two sides of an alveolus and converted into fibrin. Near the angle formed by the union of the two alveolar walls, on its lower side, an epithelial cell can be seen somewhat swollen but still attached by its extremities to the wall of the alveolus. Behind this cell only clear serum is present, for as yet it has not found its way into the lumen of the alveolus. An earlier stage in desquamating epithelium can be seen at the right of this cell on the upper surface of the alveolar wall, and still further to the right three cells can be seen slightly raised from its surface and somewhat swollen. The condition of the exudate at this point is exceedingly interesting. If the photomicrograph be examined by means of a strong reading glass it can be seen that extending through the alveolar wall, into the space beneath the middle epithelial cell, there is a delicate film of fibrin. Had these three cells been completely detached, a pore would have been formed at this point and a thread of fibrin would have extended through the alveolar wall from the lower alveolus into the upper alveolus.

Fig. 3 presents quite a different picture. Here the exudate has escaped from a very definite point. One can see where the epithelium has given way and allowed the exudate free exit into the alveolus. The epithelial cells themselves can be seen attached to the strands of fibrin as they leave the alveolar wall. If the epithelium on the opposite side of the alveolar wall, from that through which the exudate has escaped, be examined it will be found that, although in some places the epithelium has been shed off, directly over the place where the exudate found its exit the epithelium is present although the cells are slightly swollen. Although the amount of exudate is large, the injury to the alveolar wall is of such a nature that no pore has been formed.

In Fig. 4 opposite sides of the alveolar wall have been denuded of

epithelium and a well marked pore has been formed with a thread of fibrin passing through it. As in Fig. 3, epithelial cells can be seen attached to the fibrin thread close to the point where it passes through the alveolar wall.

RECAPITULATION.

Evidence is seen in Fig. 1 that the alveoli are lined with a continuous epithelium.

In Fig. 2 there has been extensive shedding of the epithelium and broad sheets of fibrin are attached to the alveolar wall. On the right the exudate has escaped from both sides of the alveolar wall and the network of fibrin has begun to appear beneath the epithelium on its upper side. Owing, however, to the fact that the epithelium is still intact, though slightly raised from the surface, no pore has been formed.

In Fig. 3 the exudate has found its exit from a very definite point and has been converted into fibrin which radiates into the alveolus. No pore has resulted because the epithelium on the opposite side of the alveolus is still intact.

In Fig. 4 the epithelium has been pushed off from diametrically opposite sides of the alveolar wall and a pore has been formed with a thread of fibrin passing from one alveolus to an adjoining alveolus.

Pores are as numerous in edema due to mitral stenosis with insufficiency of the valves as in pneumonia, but there are no threads of fibrin to direct attention to them.

This study confirms my previously expressed opinion—pores are *not* normal, preformed openings.

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5. Miller, W. S., The reticulum of the lung. I. Its relation to early tuberculous processes, *Am. Rev. Tuberc.*, 1923, vii, 141.
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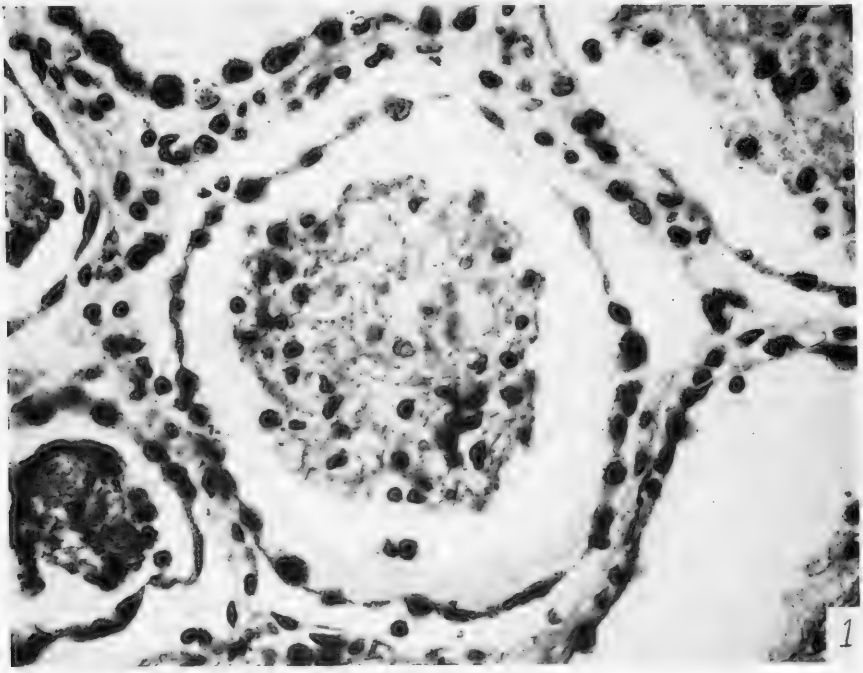
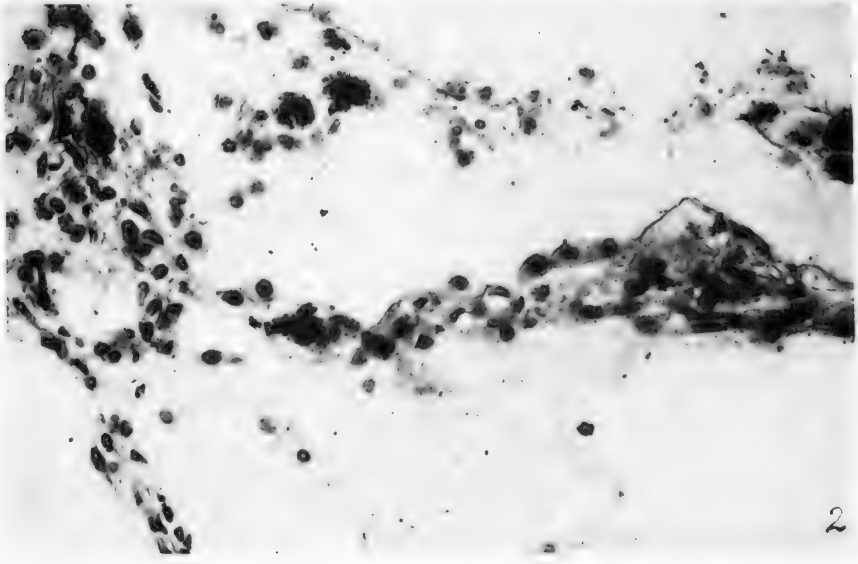
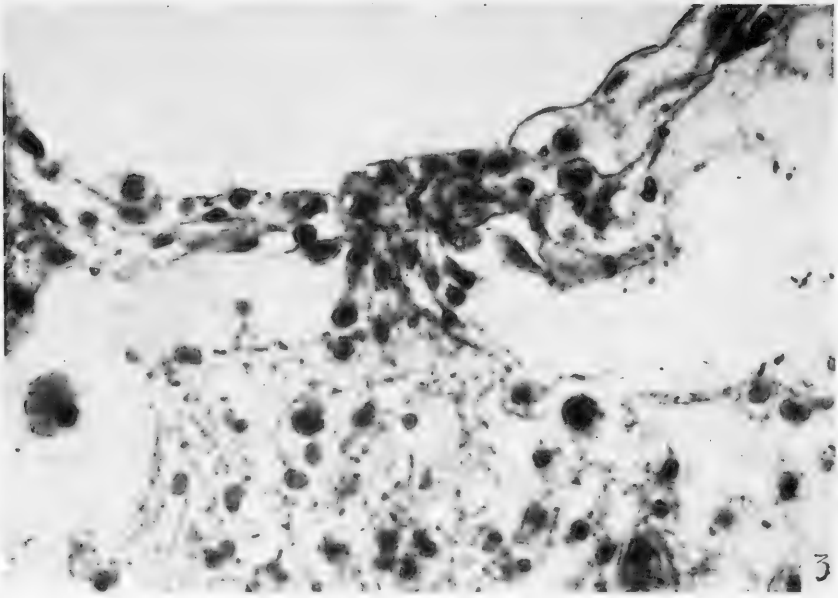
EXPLANATION OF PLATES 41 AND 42.

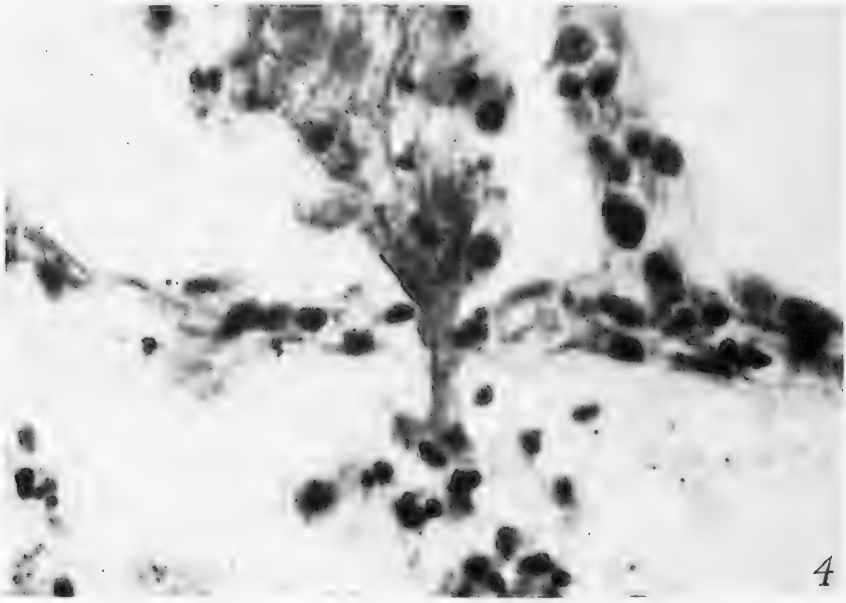
All the photomicrographs are from untouched negatives. For explanation of the figures see text. Figs. 1 and 2, × 500. Figs. 3 and 4, × 800.





(Miller: Alveolar pores of pneumonia.)





(Miller: Alveolar pores of pneumonia.)

SKIN REACTIONS TO ALTERNATE HEAT AND X-RAY EXPOSURES.*

By JAMES A. HAWKINS, Ph.D., and HARRY CLARK, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 27, 1925.)

We have previously reported some observations on the effect of simultaneous exposures to heat and x-rays on the skin reactions of guinea pigs.[1] The dosage of x-ray used in these experiments when given alone was not sufficient to cause even a mild erythema, while the heat exposure alone gave only a slight burn in 50 per cent of the animals. However, when the two agents were applied simultaneously, well marked burns resulted which healed very slowly. The time at which the burn developed and the character of the first stages were similar to a simple heat effect but the later appearance of the lesions and the slowness with which they healed suggested an x-ray burn.

It was the purpose of the present investigation to determine whether or not one of the physical agents mentioned is capable of sensitizing the tissues to the action of the other.

Method.

The character of the x-ray and the method of application have been previously described.[1,2] During exposure the animal and x-ray tube occupied separate compartments of a lead-lined cabinet with an aperture for the passage of the rays, closed with thin bristol board. Both chambers and the partitions were kept cool and ventilated by means of an electric fan. These precautions prevented any heat from the tube penetrating to the animal. The dosage used throughout the experiments was supplied by an outfit known to produce x-rays remarkably constant both in quality and intensity. The tube was operated at 30 kilovolts

*This investigation was carried out by means of funds from the Rutherford Donation.

[1] Hawkins, J. A., and Clark, H., *J. Exp. Med.*, 1925, xli, 761.

[2] Clark, H., and Sturm, E., *J. Exp. Med.*, 1924, xl, 517.

and 22 milliamperes with a target distance of 27.5 cm. The duration of exposure was 11 minutes, giving a dosage which previous experiments had proved to be suberythemal.

The technique for holding the animals in place and at a definite target distance has already been described.[2]

Heat treatments were given by means of a flat hollow brass button, 3/4 inch in diameter and 3/8 inch thick, through which water from a constant temperature bath was passed. The circulation from the bath through the button and back again to the bath was maintained by means of an electric pump driven at a definite speed. The temperature of the water fell less than 0.5°C. in passing through the circuit. When in use the button was attached firmly to the abdomen of the animal by means of a broad elastic band which encircled the body. The heat treatment was thus confined to the portion of skin covered by the button. Throughout the experiments the temperature of the water in the button was maintained at 46°C.

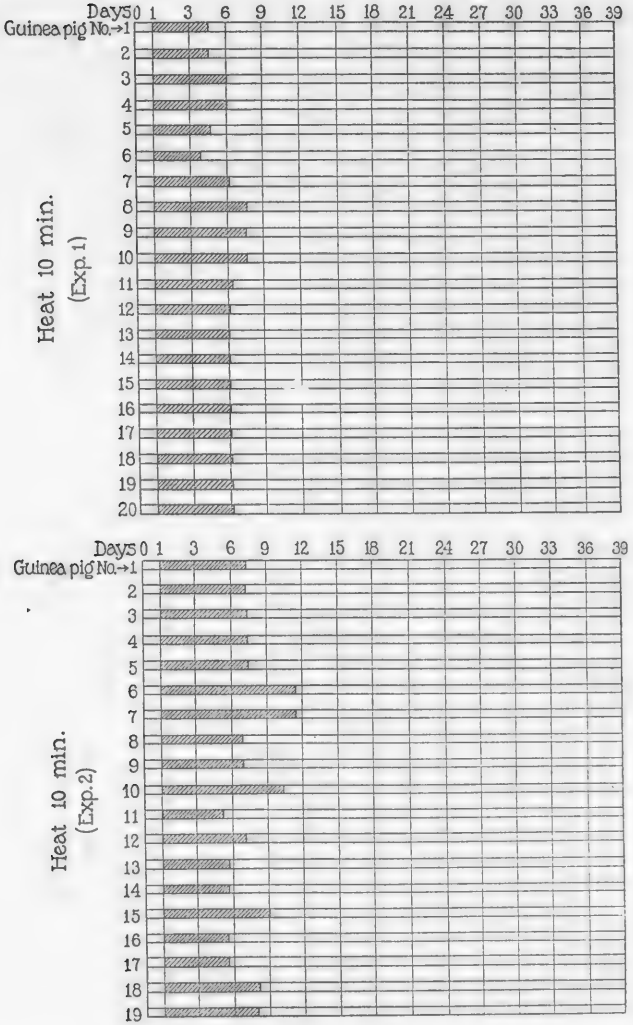
Experiment 1.—The abdomens of twenty guinea pigs were shaved and an area exposed for 11 minutes to x-rays of the intensity noted above. The brass button was then placed over part of the same area and water at 46°C. was circulated through it for 10 minutes. The heat in the same dosage was next applied to another area on the same animal but this area had received no previous exposure to x-rays. Thus the control exposures to x-rays and heat alone and the combined exposures to the two agents were made on each animal.

A slight scaling of the skin resulted from the heat exposure, which appeared about the 2nd day and lasted from 4 to 8 days (Text-fig. 1). In the areas exposed first to x-ray and then to heat scaling developed on the 2nd day and definite burns appeared on the 4th. The healing of these burns was somewhat irregular. In sixteen pigs it was completed between the 15th and 19th days, but in the remaining four the lesions were not covered till between the 32nd and 39th days (Text-fig. 2). The areas exposed to x-ray alone developed slight scaling of the skin on about the 7th day after treatment and this persisted for from 4 to 5 days (Text-fig. 3).

Experiment 2.—Heat was applied for 10 minutes to two widely separated areas on the abdomen of each of nineteen guinea pigs by means of the brass disc with the water circulated at a temperature of 46°C. Then an area on the abdomen was exposed to x-radiation so that one of the previously heated spots was included in the x-rayed area. The dosage was the same as that used in Experiment 1. Thus we had on each animal an area exposed to heat alone, x-ray alone, and one exposed first to heat and then to x-ray.

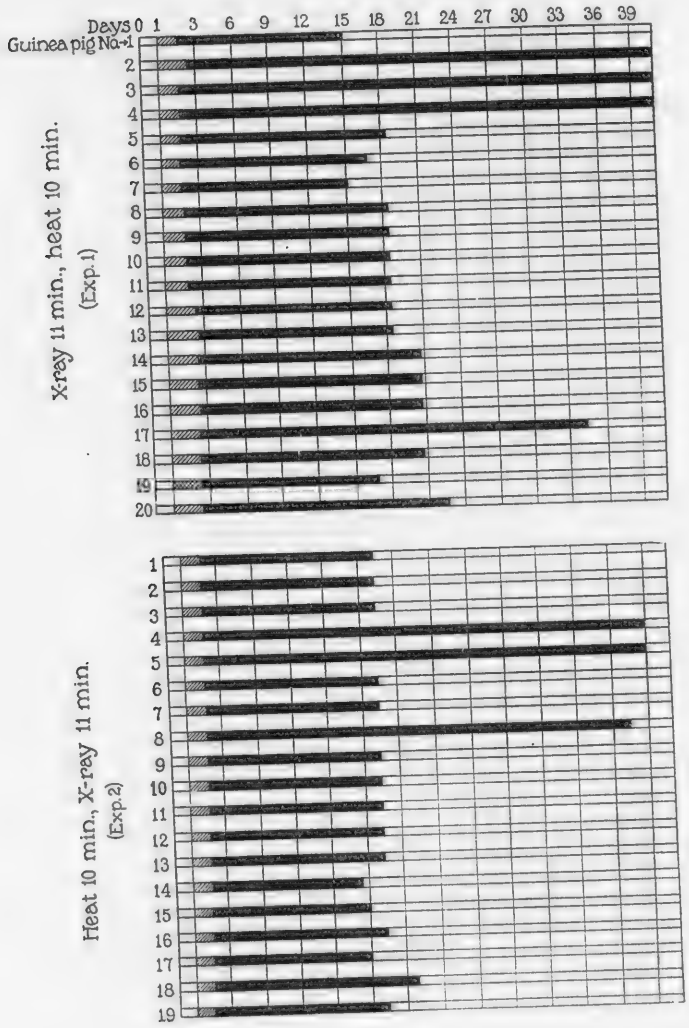
The results obtained in this experiment were identical with those in Experiment 1 and are shown in Text-figs. 1 to 3.

From these experiments it is seen that there is a very marked difference in the reaction of the areas which received either x-ray or heat alone and those exposed to both types of radiation in sequence. The maximum effect of either agent alone was a slight scaling of the



TEXT-FIG. 1.

TEXT-FIGS. 1 TO 3. Each line represents the history of a single individual area from the time of exposure until the end of the experiment. The heavy lines indicate burns; the cross-hatch indicates scaling but otherwise intact skin; and the unshaded area indicates that there was no reaction or else a healed lesion in the skin.


Days 0 1 3 6 9 12 15 18 21 24 27 30 33 36 39
Guinea pig No. 1
2
3
4
5
6
7
8
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10
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14
15
16
17
18
19
20
X-ray 11 min., heat 10 min.
(Exp. 1)
1
2
3
4
5
6
7
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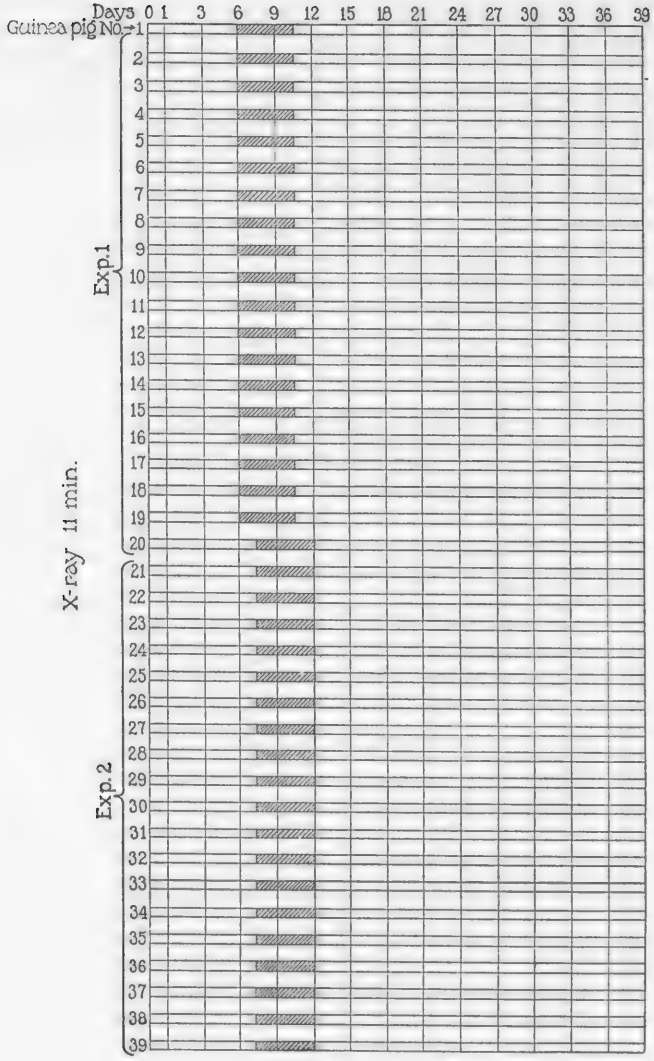
17
18
19
Heat 10 min., X-ray 11 min.
(Exp. 2)

TEXT-FIG. 2.


Days 0 1 3 6 9 12 15 18 21 24 27 30 33 36 39
Guinea pig No.
Exp. 1
Exp. 2
X-ray 11 min.

TEXT-FIG. 3.

skin. In the areas exposed to both agents a burn invariably resulted. It seemed to make no difference in the intensity of the reaction whether the heat was followed by the x-ray exposure or the x-ray followed by the heat exposure. The burns appeared as white spots corresponding in size to the area covered by the brass disc. They became evident on the 2nd day after treatment and broke down usually on the 4th day. The majority of the lesions healed in from 15 to 18 days, leaving a thick scar. In about 50 per cent of the animals, some weeks later, the areas broke down a second time with the formation of chronic ulcers which healed very slowly. It is apparent from these observations that either type of radiation is capable of augmenting the action of the other.

DISCUSSION.

The mechanism in the cell which brings about the response to radiant energy is not understood. Bovie,[3] in the discussion of his observation that ultra-violet light sensitized paramecium to heat, suggests that the radiation induces some rearrangement of the atoms within the cell so that they may change from a passive to an active stage. The process of rearrangement by which the complex cell protoplasm returns to the normal equilibrium after x-ray exposure is undoubtedly slow. The heat radiation may intensify the atomic chaos so that the cell is destroyed before adjustment can take place. In the case of the present results the reaction was the same regardless of whether the heat or x-ray exposure was made first, in which respect they differ from Bovie's observation that ultra-violet light sensitizes to heat applied later, whereas the application of the two agents in reverse order is without effect.

The nature of the lesions produced in our experiments suggests the effect of both agents. The fact that they develop so soon after treatment and their general appearance during the first few days give them a close resemblance to a pure heat effect, but the later appearance of the burns, the slowness of healing, and the type of resulting scar are more characteristic of an x-ray effect. The observations would seem to be capable of further analysis with a possibility of yielding some insight into the nature of the effect of radiant energy on the living cell.

[3] Bovie, W. T., and Daland, G. A., *Am. J. Physiol.*, 1923, lxvi, 55.

SUMMARY.

Different areas on the abdomen of the same guinea pigs have been exposed to suberythema doses of soft x-rays, to heat of an intensity below the critical dose for the production of burns, and to both radiations in sequence.

The only effect of exposure to x-ray or heat alone was a slight scaling of the skin. The areas exposed to heat and x-radiation developed well marked and persistent burns. The results were the same no matter in which sequence the agents were applied.

STUDIES ON EXPERIMENTAL RICKETS.

I. The Occurrence of Rickets in Young Rabbits Born of Mothers Infected with Schistosomum japonicum.

By R. KAWAMURA, M.D., and Y. KASAMA, M.D.

(*From the Pathological Laboratory of Niigata Medical University, Niigata, Japan.*)

Plates 43 and 44.

(Received for publication, June 4, 1925.)

In spite of the fact that rickets can be produced by a variety of experimental methods, and this disease can be cured or prevented, the real mechanism by which rickets is produced remains uncertain. Believing, therefore, that any new method or condition which can cause this disease may be a matter of great interest, we wish to record here our findings.

A parental influence on the predisposition of the young to this disease was suggested as early as the seventeenth century by Glisson (1668). Since then, numerous investigators have shown either by statistics or by experiments, that certain maternal nutritive factors such as calcium, phosphate, and the antirachitic vitamin can exert a considerable influence on the generation following after. Byfield and Daniels (1923) state that in a series of animal experiments, it has been possible to produce gross rickets by faulty diet into the second generation only.

During the course of our investigations on the study of certain parasitic diseases, we have accidentally encountered rickets in young rabbits born of mothers infected with the parasites. The preliminary report of this investigation was made before the Japanese Pathological Meeting in 1922.

EXPERIMENTS.

Female rabbits were infected with *Schistosomum japonicum* by rubbing on the well shaven and scarified skin of their abdomen the cercariæ, obtained by emulsifying a fresh water snail, *Katayama*

nosofora robsoni. Some of these infected females survived and became pregnant. During the whole course of the experiment, the experimental and normal control rabbits were fed with exactly the same diets, which, we had assured ourselves through other experiments in producing experimental rickets, did not give rise to this condition. To all appearances, both groups of the animals ate equally well, and no evidence of a deficiency was noted. Young rabbits born of the normal mothers showed a normal rate of growth, whereas those born of infected mothers developed signs of rickets between 25 and 35 days after birth; their growth also was considerably retarded, as shown in Table II, and the necropsy gave gross evidence of rickets. The joints of the extremities, especially of the wrist, ankle, and knee, were enlarged and thickened. The rosary at the rib joints was evident, and sometimes fractures of ribs were observed. A kyphoscoliotic disturbance of the vertebræ occurred in some cases (see Fig. 1).

TABLE I.

	Infected mother No.								
	I	II	III	IV	V	VI	VII	VIII	IX
Total offspring born	6	4	3	3	1	2	3	4	5
Rachitic young	2	1	1	3	1	2	2	4	5

Microscopically, there were typical rachitic changes, namely proliferation of the cartilage zone, thickening of unossified metaphyses, fibromatosis, and hyperemia of the bone marrow in the epiphyses. The calcium of the bones was greatly reduced (see Fig. 1, 2, and 4).

The ratios of the rachitic young to the total offspring are shown in Table I.

Of all the young born of the infected mothers, we found 67.7 per cent rachitic. In these animals, the rachitic change reached its maximum in 50 to 60 days and in 90 to 100 days it had either declined or disappeared.

In order to see whether these rachitic changes were identical with those produced experimentally in rats and dogs by various investigators, we fed some of the affected young with cod liver oil. We selected two groups of the young which were afflicted in about the same degree with rickets, as shown in Figs. 6 and 7. The right fore

paws of both groups of animals were amputated, under ether anesthesia and aseptic conditions, when they were 54 days old, and to one group, 0.2 to 0.5 cc. cod liver oil was given daily thereafter, the other group being kept as a control. At the end of 27 days, both groups were killed, and the left hind paws were examined. As shown in the x-ray photographs (Figs. 8 and 9), there was no sign of rickets detectable in those young fed with the cod liver oil, while the controls were still markedly rachitic.

TABLE II.

Method of experiment.	Mother No.	Young rabbit No.	No. of young taken.	Age of animal.		No. of rachitic animals.		Cod liver oil given per day.	Weight of young animals.	
				At amputation.	At autopsy.	At amputation.	At autopsy.		At amputation.	At autopsy.
				days	*days*			*cc.*	*gm.*	*gm.*
Cod liver oil given.	IX	16 17 20	3	54	81	3	0	0.2–0.5 Total 10.	325–340	515–680
Cod liver oil not given.	IX	18 19	2	54	81	2	2	0	340–350	500–560
Control.	III	1 2 3	3	23	39 49	0	0	0	340–480	400–420

Thus it seemed evident that the rickets produced in the young born of mothers afflicted with *Schistosomum japonicum* is the same as that produced experimentally by the usual methods in other animals. Details of the experiment are given in Table II.

Since, as stated before, the experimental conditions were such that neither dietary nor environmental factors could be the direct cause of these changes in the young, it is safe to conclude that the congenital factor is responsible for this disease. The question, however, whether it is of hereditary nature or is due to a direct transmission of toxin from the parasites of the mother to the young is yet to be answered.

In order to see whether the parasites can cause rickets directly, we infected young healthy rabbits with them.

As shown in Table III, when young weighing less than 500 gm. were infected with *Schistosomum japonicum*, slight signs of rachitic change are noticeable within 13 days after inoculation. These changes reach their maximum within 30 to 60 days and gradually diminish

TABLE III.

Method of experiment.	Mother No.	No. of young taken.	No. of rachitic animals.	Time between autopsy and amputation.	Age at amputation.	After experiment.	Rachitic change at amputation.	Body weight.	
								Time of experiment.	At autopsy.
				days	*days*	*days*		*gm.*	*gm.*
Young rabbits infected with parasite.	I	4	4			24		360	500
Control.	IV	2	0			76		500	1170
Young rabbits infected with parasite.		6	6			20		320	280
Contro	III	1	0			68		550	1120
Late findings in young rabbits.	II V	6	0	30	32	97	6	170	570
Control.	VI	2	0	82	74	144		550	2350
Adult rabbits infected with parasite.	VII	3	0			82–136		1520–1700	1270–1800

TABLE IV.

Method.	Mother No.	No. of young.	No. of rachitic young.
Fed with egg yolk.	EI	3 3	2 3
	VIII	2	0
Control.	VIII	2	0

but they are detectable even after 75 days. After 90 to 140 days, however, even a microscopic examination reveals no sign of rickets. It may be added here that this parasite causes rickets only in the young, never producing this disease in adults.

In order to see whether the action of the parasite is specific or not, we have conducted a few preliminary experiments, the results of which are given in Table IV. As shown in the table, the young rabbits born of mothers fed with fowl egg yolk have rickets. From the mothers which were used in experiments in connection with the investigation of a tumor problem some of the young born became rachitic. These rachitic changes became milder as the young grew. If, however, the young rabbits are fed directly with the egg yolk, no rickets is produced. It is conceivable that the changes after feeding egg yolk may come about through the same mechanism as those induced by the parasites.

DISCUSSION AND CONCLUSION.

Our observations show that young rabbits born of mothers afflicted with *Schistosomum japonicum* develop typical rickets. Rickets can also be produced if we infect the young, healthy rabbits with the same parasite. It is natural to suppose that the rachitic changes are caused by the parasite itself. Since, however, a similar disease can be produced in the offspring, when the mother is fed on egg yolk, the causation is not limited to the action of this parasitic toxin alone.

The toxin of *Schistosoma* may disturb the calcium and phosphorus metabolism of bone in young animals, especially in the period of vigorous growth; that is, 20 to 40 days after birth of the rabbits. Or it may exhaust some element important in the calcium and phosphorus metabolism such as vitamin A or D. The fact that exhaustion of the antirachitic factor in the mother causes rickets in the young, as Grant (1924) showed, and that certain low grade infections can exhaust vitamin B as shown by Wedgewood (1924), is in line with this conception.

It may be added here that most investigations on rickets have been carried out on rats and dogs. We have found a simple and excellent way of producing rickets in rabbits by dietary deficiency. Concerning this method, we shall report elsewhere.

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EXPLANATION OF PLATES.

PLATE 43.

FIG. 1. X-ray photograph of Rabbit 1, born of mother injected with *Schistosomum japonicum*.

FIG. 2. Photomicrograph of a section of radius of Rabbit 1 (rachitic).

FIG. 3. Section of radius of Rabbit 2, born of normal mother.

PLATE 44.

FIG. 4. Section of radius of Rabbit 4 (33 days old), born of injected mother.

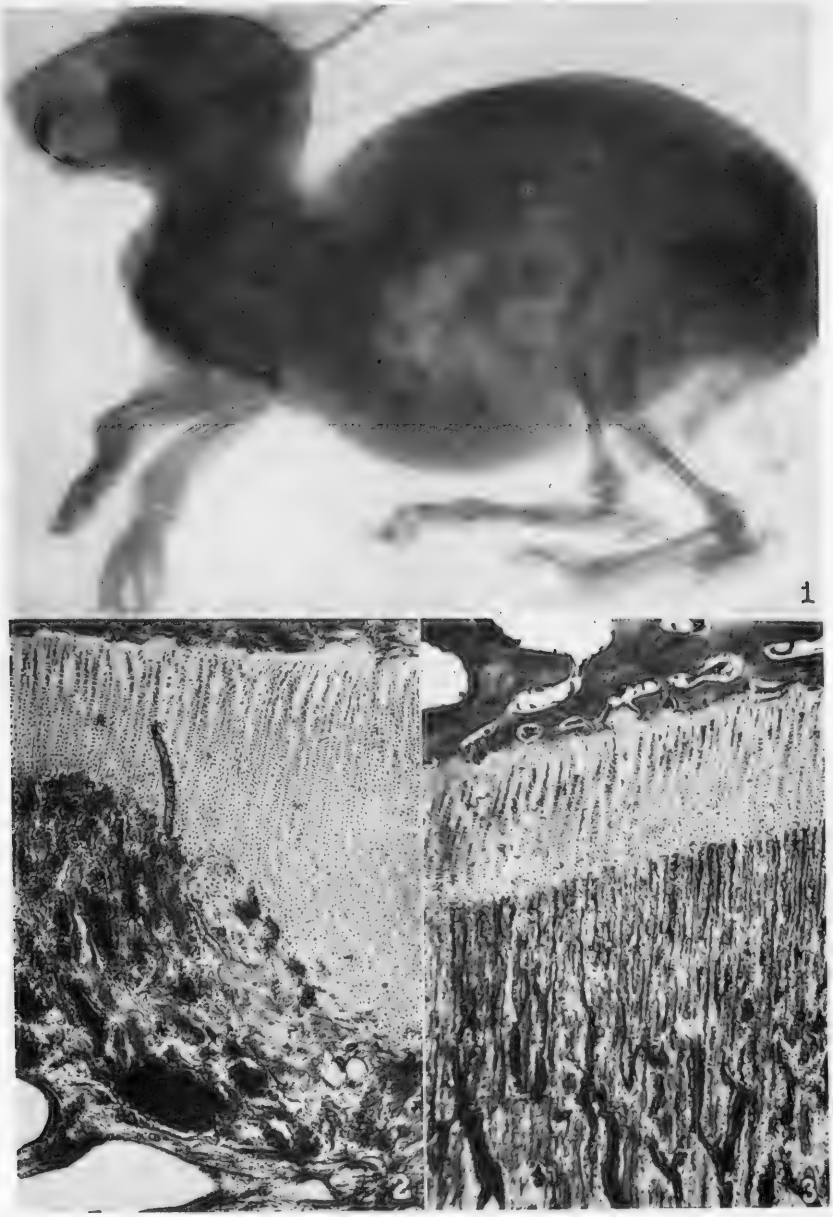
FIG. 5. Section of radius of Rabbit 5 (33 days old), born of normal mother.

FIG. 6. Right hind paw of Rabbit 20, amputated at 54 days old.

FIG. 7. Right hind paw of Rabbit 19, amputated at 54 days old.

FIG. 8. Left hind paw of Rabbit 20, fed with cod liver oil for 27 days after the first amputation (*cf.* Fig. 6).

FIG. 9. Left hind paw of Rabbit 19, 27 days after first amputation, no cod liver given (*cf.* Fig. 7).



(Kawamura and Kasama: Experimental rickets. I.)

(Kawamura and Kasama: Experimental rickets, I.)

THE PROBLEM OF THE ETIOLOGY OF HERPES ZOSTER.

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(Received for publication, August 7, 1925.)

The nature and etiology of that group of infectious diseases of which one of the features is a vesicular eruption on the skin are at the present time much confused. Rivers (1) has constructed a table indicating a possible relationship between a series of these diseases beginning with sheep-pox and horse-pox and extending through cow-pox, smallpox, varioloid, alastrim, chicken-pox, and herpes zoster to symptomatic herpes and lethargic encephalitis. Certain of these conditions resemble each other in their clinical manifestations, others have little in common. Certainly, the symptoms of herpes simplex have little resemblance to those of smallpox. The only feature present in all of them, except lethargic encephalitis, is a vesicular eruption of the skin. In most of the conditions the skin lesions show similar histological characteristics.

At one time or another some relationship in etiology between various members of the group, or even an identical etiology in all of them has been suggested. In none of the conditions has the etiological agent been cultivated, but there is considerable evidence that the responsible agent in most of them is ultramicroscopic or filterable. It is evident that in the absence of cultivation, in order to establish the etiological relation of an ultramicroscopic virus with one of these diseases, it is necessary to reproduce, in animals or man, lesions resembling the natural infection. With certain of these diseases, notably smallpox and vaccinia, the experimental reproduction of the disease is comparatively easy. With others, such as varicella and herpes zoster, all attempts to transmit them to animals have led only to equivocal and uncertain results. In the case of herpes simplex, although a virus has been isolated which is highly infectious for rabbits, the clinical picture produced is not, as will be discussed below, identical with herpes simplex in man.

Recent investigations have suggested an especially close etiologic relationship between varicella, herpes zoster, and herpes simplex. Some of the workers, mainly on the basis of clinical observations, claim an identity of the etiological agent concerned in varicella and herpes zoster, others are of the opinion that herpes zoster is due to a modified herpes simplex virus. An etiologic relationship between these conditions is also suggested by the fact that in the epithelial cells of the skin lesions in all of them acidophilic intranuclear inclusion bodies are found. These bodies were first described by Tyzzer (2) in varicella and later by Lipschütz (3) in herpes simplex and herpes zoster.

Inclusion bodies, both intranuclear and extranuclear, have been studied in great detail by Lipschütz, and are considered by him to be characteristic of diseases of the filterable virus group. Goodpasture (4) has corroborated and extended Lipschütz's studies of these structures in connection with herpes simplex and agrees with Lipschütz as to the specific nature of intranuclear inclusion bodies. Rivers and Tillett (5) have demonstrated similar intranuclear inclusion bodies in the corium of skin lesions associated with a rabbit virus isolated by them. In the study of diseases of this group, the finding of these characteristic nuclear changes in experimental lesions in animals or man is therefore of importance in determining whether the reaction obtained is specific. Following the observation by Grüter (6) in 1920 and those of Löwenstein (7, 8) investigators in all parts of the world have demonstrated that the virus of herpes simplex, when inoculated into the scarified cornea of rabbits produces with great regularity a vesicular eruption followed by an intense keratoconjunctivitis. Inoculations into the skin less frequently give rise to lesions. As Doerr and Vöchting (9) first observed, corneal inoculations are frequently followed by marked nervous symptoms and death and similar symptoms can be produced by direct inoculation into the brain. The inoculations of the virus into rabbits, therefore, gives rise to lesions which *may* resemble those seen in man, but in most cases the lesions and symptoms differ both in character and severity from those present in the mild and common condition in human beings known as herpes simplex. It is of importance, however, that in all the lesions produced in animals, including those in the cornea, the skin, and the brain, the most characteristic feature of the lesion of herpes simplex in man is reproduced; namely, the occurrence of cells containing intranuclear inclusion bodies. The intracutaneous inoculation of the vesicular fluid of herpes simplex either into an individual already infected with herpes simplex or into a normal person has not given as constant results as has the inoculation of this fluid into the rabbit's cornea. Man's susceptibility to herpes simplex seems to depend on certain secondary factors which are, at the present time, unknown.

The successful inoculation of rabbits with herpes simplex material

was followed by attempts to transmit varicella to animals, but so far these attempts have not been successful. (For a review of the literature, see Rivers and Tillett (1).) Many attempts have been made to reproduce varicella in man by inoculating material from active cases into normal individuals. Kling (10) reported the successful vaccination of children against chicken-pox by inoculation with vesicular fluid. Certain later observers employing the method of Kling have noted the development of a local vesicle or papule at the site of inoculation, others have described the occurrence of a generalized eruption (true chicken-pox?), others have stated that no obvious lesions result from the inoculation. So far as we are aware no histological study has been made of any of the lesions described. The difficulty in successfully inoculating animals or man with varicella virus is of interest in view of the claims which have been made regarding the identity of the viruses of chicken-pox and herpes zoster.

Inoculation of Virus of Herpes Zoster into Animals.

Prior to 1921, attempts to inoculate animals with herpes zoster had proved negative. In this year Lipschütz (3) reported successful results.[1] Seven cases which were apparently typical as regards clinical manifestations were studied. The vesicular fluid was obtained early in the disease and was rubbed into the scarified cornea of rabbits. In certain instances the fluid was combined with the "roofs" of vesicles. Lipschütz considers that positive results were obtained with the material from four cases. In view of the importance of his conclusions a brief review of his cases will be given.

Case I.—It is stated that the inoculation of the material into the rabbit's eye was followed in 4 days by a slight opacity of the cornea along the lines of scarification. The eye was removed and sections were made through the cornea. Occasional giant epithelial cells were present along the lines of scarification. Under Bowman's membrane hypertrophied and swollen connective tissue cells

[1] No attempt will here be made to review the entire literature concerning the inoculation of animals with material from cases of herpes zoster. Attention will be drawn chiefly to those authors whose results have been considered suggestive in contrast to those who have obtained definitely negative results. A complete review of the literature relating to herpes has recently been published by Doerr (34, 25).

were seen but no leucocytes. In the nuclei of the epithelial cells and also of the swollen connective tissue cells there were to be seen occasional, typical, round, sharply circumscribed and clearly demonstrable intranuclear inclusion bodies.

Cases II and III.—These were also early cases and material from them was similarly inoculated into the eyes of four rabbits. In one of the rabbits after 4 days the cornea showed an intense circumscribed keratitis, with the appearance of a slightly elevated "*eitrig getrübten*," and "*daherweiszlich*" appearing vesicle. The microscopic examination of this eye showed marked infiltration with pus cells. No inclusion bodies were found. In another animal a keratitis developed, but the occurrence of vesicles or the presence of inclusion bodies is not noted. The results in the other two animals were negative. In these two cases, therefore, the evidence presented in the protocols which indicates positive results is very slight.

Case IV.—This was also a typical early case of herpes zoster. Vesicular material from this case was inoculated into the eyes of two rabbits and two guinea pigs. The results in the two guinea pigs and in one of the rabbits were negative. In the second rabbit the inoculated eye showed on the 2nd day conjunctivitis and circumscribed corneal infiltration. The eye was enucleated on the 4th day and in microscopical sections very numerous intranuclear inclusion bodies were found in the epithelial cells.

The remaining three cases were studied at later periods of the disease and the results were negative.

The experiments of Lipschütz with material from these seven cases can, therefore, as judged from his brief protocols, be considered to have yielded positive results in only two animals, and in these instances the results are of importance chiefly on account of the presence of intranuclear inclusion bodies in the epithelial cells.

Lipschütz (11) considers that his positive findings have been confirmed by Marinesco and Draganesco, Truffi, Mariani, and Blanc and Caminopetros, and that the successful transmission of herpes zoster to animals has thus been accomplished. It is therefore important to review in some detail the reports of these investigators.

Marinesco (12), and Marinesco and Draganesco (13), injected material from three cases of herpes zoster.

Case I.—Herpes zoster localized on the thigh. Vesicular fluid was inoculated into the scarified corneas of four small rabbits, and into the second cervical ganglion of two small cats. The rabbits all remained unaffected. The ganglia of the cats were examined after 7 days and in one of them there was evident lymphatic infiltration and atrophy of the neurones.

Case II.—Herpes zoster of the first and second branches of the trigeminus nerve. Since the vesicles contained but little fluid, spinal fluid obtained on the 6th day of the disease was used for inoculation. The injections were made into the anterior

chamber of one eye, and into the scarified cornea of the other eye in each of nine rabbits. Moreover, in five of these nine rabbits, in addition to the eye inoculations, 0.2 cc. of spinal fluid was inoculated intracerebrally. The eyes of the first three rabbits showed only injection due to injury. On the 4th day, in Rabbit 4, in the cornea of the eye in which the injection was made into the anterior chamber, there was noted a zone of infiltration. Rabbit 5 showed two points of infiltration on the scarified cornea. Rabbits 6, 8, and 9 were negative. Rabbit 7, besides a febrile reaction on the 4th day, developed an area of infiltration reaching the center of the pupil. The emulsified brain and cerebellum of this rabbit were inoculated into the cornea of four more rabbits, two of which showed on the 3rd and 4th days grayish infiltration along the lines of scarification. No statement is made concerning microscopic examination.

So far as can be judged from the protocols, therefore, inoculations made with the material from these two cases produced no characteristic lesions. A macroscopic infiltration of the cornea can hardly be regarded as specific.

Case III.—Herpes zoster lesions on the thigh. Vesicular fluid was inoculated into the scarified cornea of three rabbits, two of which on the 4th day showed a linear infiltration. The writer states (12) that the sections of the cornea of one of these rabbits showed swollen, edematous cells, and that here and there could be seen the "specific nuclear lesion, consisting in atrophy of the chromatin which is pushed toward the membrane, while the acidophilic mass, which has developed, offers a striking resemblance to the inclusions described by Lipschütz in animals injected with herpes." It is undoubtedly on this last statement that Lipschütz bases the view that his own observations have been confirmed by Marinesco and Draganesco. Certainly nothing else in the protocols indicates the occurrence of a specific lesion.

Truffi (14) studied three cases of herpes zoster. The results in the first two were negative. Vesicular fluid from the lesion of Case III, cervicobrachial in distribution, was obtained on the 3rd day of the disease and inoculated into the scarified cornea of one rabbit. After 48 hours a slight opacity along some of the lines of scarification, and an intense conjunctivitis were noted. The corneal opacity disappeared rapidly and the eye regained its normal appearance by the 7th day. 22 days after inoculation the rabbit showed symptoms of encephalitis and was killed 10 days later. The microscopical examination of the brain was negative. The presence of intranuclear inclusion bodies in the brain cells is not noted. The inoculation of the brain emulsion into the scarified corneas of two rabbits and two guinea pigs failed to produce lesions.

Most of the attempts made by Mariani (15) to inoculate the cornea of rabbits with herpes zoster resulted negatively. In one instance he obtained a very acute keratoconjunctivitis with hypopyon and purulent ophthalmia. In only one case did there result a keratitis which he was able to transmit in series. The lesion produced was clinically and symptomatically very similar to the keratitis produced by herpes simplex virus. No description of the case of herpes zoster from which

the material for inoculation was obtained, is given. No statement concerning microscopic examination of the corneas is made. Mariani himself considers this single experiment inconclusive.

Material from nine cases of herpes zoster was inoculated by Blanc and Caminopetros (16) into the eyes, cornea, conjunctiva, skin, brain, and spinal cord of a series of animals, including rabbits, mice, sheep, pigeons, monkeys, and a dog. Three monkeys (*Macacus rhesus*) were inoculated as follows: one into the eye, one into the spinal canal, and the third into the skin of the thoracic region which had previously been shaved and excoriated. The inoculations in the first two monkeys resulted negatively. The third monkey showed a slight inflammatory reaction at the site of inoculation but recovered without the appearance of vesicles. All the other animal experiments gave negative results with the following exceptions; two rabbits developed a late paralysis which, however, the authors considered was probably not specific, and one rabbit and one sheep, both inoculated with material from the same case, developed a definite keratitis, which spread from the point of inoculation. The writers think that this lesion might easily be interpreted as a reaction resulting from the injection. They conclude that the problem of the transmission of herpes zoster to animals remains open and they apparently consider their own experiments negative or inconclusive.

Meineri (17) claims to have produced encephalitis in a guinea pig by the intracerebral inoculation of vesicle fluid from a case of herpes zoster. A careful analysis of his experiments, however, in our opinion, indicates that his findings can best be interpreted as the result of trauma. The writer also injected vesicle fluid obtained on the 3rd day of the disease from one of his cases of herpes zoster into the skin of the arm of the patient and into the skin of a normal man. These injections in both instances were without visible result.

The review of the publications of those writers whom Lipschütz quotes as having confirmed his work shows that two of the writers regard their own results as inconclusive. Only Marinesco and Draganesco found microscopical lesions which might be interpreted as specific.

On the other hand, many other authors report entirely negative results following the inoculation of herpes zoster material into the sacrified corneas of rabbits: Kraupa (18); Baum (19); Löwenstein (8); Teissier, Gastinel, and Reilly (20); Kooy (21); Netter and Urbain (22); Bloch and Terris (23); Simon and Scott (24); and Doerr (25).

It is evident, therefore, that the results of attempts to inoculate animals with material from cases of herpes zoster must be considered at present to be inconclusive.

Herpes Simplex and Herpes Zoster.

Although it has not been possible to demonstrate conclusively any specific virus associated with herpes zoster, certain writers have presented evidence which suggests that, in certain cases at least, the symptoms and lesions of herpes zoster may result from the presence of the virus of herpes simplex.

Luger and Lauda (26) have published several papers on the problem of the etiology of herpes zoster. In their first paper they give the results obtained by inoculation with material from seven cases of typical herpes zoster, employing the technique used by Lipschütz. In none of the eyes inoculated did any macroscopic reaction occur. On microscopical examination there was found fairly regularly edematous swelling of the epithelial cells, giant cell formation, and "*ballonierende*" degeneration, but in no instances were cell inclusions or characteristic changes of the nuclei seen. They themselves considered the results in this series of experiments negative. This paper was followed by two others (26) in which two more cases, considered by the authors to be typical cases of herpes zoster, were studied. From both of these latter cases these investigators were able to isolate a virus, infectious for rabbits, which they showed, by immunity experiments, to be identical with the virus of herpes simplex. The first case occurred in a patient suffering from acute epidemic encephalitis, and the eruption was localized in the gluteal region. The involved area was not large and the clinical diagnosis of herpes zoster in this case must be considered doubtful. A typical herpes simplex virus was isolated without any difficulty. In the second case the eruption was thoracic in distribution and the case was clinically typical. On the 8th day of the disease, some vesicle fluid was inoculated into the eye of one rabbit. No local reaction occurred. The 10th day after inoculation the animal developed encephalitis and died on the 11th day. For reasons not stated, the glycerolated brain of this animal was stored on ice for 11 days before further inoculations were made. Inoculations then made with this material into the brains and eyes of rabbits produced the characteristic reactions of a herpes simplex virus. The authors conclude from these results that herpex simplex virus may be isolated from herpes zoster vesicles. They offer no explanation of the fact that although the virus failed to produce any reaction on the cornea, it nevertheless possessed the ability to invade the brain.

Grüter (27) inoculated material from three cases of herpes zoster into the scarified corneas of rabbits. A mild keratitis resulted. No detailed description of the lesion or results of microscopic examination are given. Grüter, however, believes the lesion obtained was specific and attributes it to herpes simplex virus of a low grade of virulence. He states that there is no evidence for assuming a specific virus for herpes zoster. The data presented, however, are not sufficient to establish the isolation of a true herpes simplex virus from these cases.

Bastai and Busacca (28), in a general article on herpes, state that they inoculated material from three cases of herpes zoster into the cornea of rabbits and into the cornea of one monkey (*Macacus*). Rabbits were also inoculated intracerebrally. None of the animals showed any reaction, with the exception of one rabbit which developed a slight keratitis. No attempts were made to transmit this lesion, and no microscopical examinations are reported. These authors also are of the opinion that herpes zoster is probably a manifestation of infection with herpes simplex virus. The experimental data presented, however, are hardly sufficient to justify this point of view.

Cipolla (29) is of the opinion that the cases of herpes zoster should be divided into two groups, zoster symptomaticus and zoster idiopathicus, although he states that there is great difficulty in drawing any sharp line of division. He has studied four cases which he considered zoster idiopathicus and three cases which he called zoster symptomaticus.

The results of inoculation of material from the so called idiopathic cases were as follows: In one rabbit and two guinea pigs there occurred "a keratitis definitely evident along the lines of scarification but without vesicle formation and a hyperemic conjunctivitis, with redness and slight seromucous exudation." The results in all the other animals inoculated were negative. No note is made of any microscopical examinations.

Of the cases which he considered symptomatic, in one the eruption followed an injury, in the second it was associated with a tuberculous pleurisy, and in the third the patient was taking bromides and the herpetic eruption was considered to be a manifestation of bromide intoxication. In all these cases the eruption was on the chest. In two cases the appearance of vesicles was preceded by acute neuralgic pains, in the third case no note is made concerning pain, but the eruption was accompanied by a febrile reaction. The inoculations with material from all these cases gave rise to severe keratitis and conjunctivitis resembling that caused by the virus of herpes simplex. In one rabbit an encephalitis developed. No statement concerning microscopical examinations is made.

In our opinion the three so called symptomatic cases must be considered from the clinical features as probably cases of herpes zoster. The comparatively trivial associated injuries (trauma or drug) might easily have been overlooked and then the diagnosis would not have been questioned. It is of great interest that the animal experiments indicate that in these cases a virus indistinguishable from that of herpes simplex was present in the lesions.

Teague and Goodpasture (30) were able to produce zoster-like lesions in the skin of rabbits and guinea pigs by the inoculation of herpes simplex virus into areas of the skin previously treated with coal tar. The study of the corresponding posterior root ganglia showed lesions comparable to those found in man in the ganglia innervating the area of zonal eruption.

The writers do not maintain that they have reproduced the human disease herpes zoster in animals, but they believe there is a close analogy between the experimental condition produced by them and true herpes zoster. In their

opinion the herpes simplex virus first multiplies at the site of inoculation in the skin and passes up the corresponding spinal nerve to its spinal ganglion; the virus then seems to pass centrifugally along the nerve and its branches to the skin, where it multiplies rapidly and gives rise to characteristic herpetic vesicles. They draw attention to the difficulty of sharply separating cases of herpes simplex and herpes zoster and discuss the occurrence of intermediate cases. From a case of the intermediate type inoculations were made into the tarred skin of a rabbit. A zonal eruption, as described above, resulted. They feel that the evidence presented strongly suggests that the virus of herpes zoster is closely allied to the virus which causes herpes simplex, probably differing only in virulence.

The interesting hypothesis presented by Teague and Goodpasture (30) and by Luger and Lauda (26) concerning the relation of herpes zoster to herpes simplex does not find acceptance, however, by Lipschütz (31). He emphasizes the point of view that in the production of an experimental herpes zoster it is of prime importance that the starting point be a typical clinical case and not a border line case.

At the present time the evidence that herpes zoster may result from infection with herpes simplex virus rests upon the isolation of a virus apparently identical with that of herpes simplex from a small number of cases. No description of the type of case from which the material employed for inoculation was obtained is given by Grüter or by Bastai and Busacca. The case described by Teague and Goodpasture and the first case described by Luger and Lauda belong to the intermediate type of cases. The second case of Luger and Lauda, and the so called symptomatic cases of Cipolla, seem to have been clinically typical cases of herpes zoster. It is possible, therefore, that in certain instances the virus of herpes simplex may be isolated from cases clinically characteristic of herpes zoster, but the evidence for this is not complete and the conclusion that herpes zoster may be the result of infection with herpes simplex virus needs further verification.

Varicella and Herpes Zoster.

Several observers (Lipschütz, Meineri, and others) have made isolated attempts to inoculate human volunteers with herpes zoster, but always with negative results. Recent studies of Kundratitz (32) seem to show that herpes zoster can be successfully transmitted to very young children. This author wished to test out von Bókay's (33) hypothesis, based on clinical observation, that the virus of varicella, under certain unknown conditions, may produce a typical picture of herpes zoster and that the virus from this lesion may in turn cause varicella. He therefore

attempted to immunize children against varicella by the inoculation of material from herpes zoster cases. His first results were negative, but his later attempts proved successful. He now reports that he has inoculated material from ten typical cases of thoracic herpes zoster and has had positive results with the material from five of these cases. Positive reactions were obtained only in children under 5 years of age. Children who reacted positively were subsequently shown to be immune to varicella.

Kundratitz's work seems to indicate that the virus of varicella and that of herpes zoster are identical or, at least, closely related. It is unfortunate that Kundratitz does not give a description of the cases of herpes zoster used by him for inoculation. It would be interesting to know whether there were any clinical differences between the five cases of herpes zoster with which he was able to make successful transfers and the five cases in which transfers resulted negatively for, as von Bókay and others have shown, the vesicles of varicella may be quite localized, resulting in lesions resembling herpes zoster. The relation between herpes zoster and varicella will, in all probability, not be entirely cleared up until we are able to transmit either one or both of these diseases to animals.

EXPERIMENTAL.

Nine cases of herpes zoster have been studied by the writers and inoculations have been made into a series of animals. The following are abstracts of the histories and protocols of the experimental studies.

Case I.—A. M. Age 13. Patient admitted to the hospital Oct. 4, 1924, suffering from subacute rheumatic fever and chronic cardiac disease. She gave no history of a previous attack of herpes zoster or chicken-pox. The arthritis had almost entirely disappeared and the cardiac lesion was well compensated, when on Dec. 6, 1924, the patient complained of pain and itching over the upper scapular area, in the axilla, and posterior part of the upper arm. On examination of this area there was discovered a rash consisting of small, discrete papules and vesicles distributed in patches over a zone on the upper chest from the midline behind to the midsternal line in front, and over the inner and posterior surface of the arm. The area of distribution corresponded to Head's second and third dorsal areas. During the following days the vesicles became larger. The temperature was not higher than 99.8° until Dec. 11, when some of the vesicles had become pustular, and now the temperature rose to 101.4°. The pain was severe and characteristic of herpes zoster, and the appearance and distribution of the lesions were typical. A small piece of skin was removed and microscopical sections showed characteristic vesicles with numerous intranuclear inclusion bodies in the epithelial cells.

On Dec. 9, the 3rd day of the disease, fluid was pipetted from a number of vesicles and a small piece of the involved skin was obtained. The skin was ground between two glass slides, the ground material was washed off in a small amount of normal saline solution and was added to the vesicular fluid. Small amounts of this emulsion were rubbed into the scarified[2] corneas of two rabbits, Nos. 1 and 2, and also injected intracutaneously into the shaved skin of Rabbit 2 and into the skin of Guinea Pig 1. The area of skin in the guinea pig where the injection was made had been painted several days previously with coal tar solution. The emulsion was also rubbed into the scarified skin of Rabbit 3, which had received one painting of tar 5 days before, and into the scarified skin of a similarly tarred guinea pig, No. 2. (The rabbit and guinea pigs were painted with a refined coal tar solution obtained through the courtesy of Dr. Jas. B. Murphy. This refined coal tar was much less toxic than ordinary tar and could be applied in a fairly thick coat, so that one painting resulted in a marked reaction.) On the following day, Dec. 10, vesicular fluid was again obtained from fresh vesicles and also another piece of skin. This material was treated in the same way as that obtained on the preceding day, and inoculated in the following ways. The cornea of Rabbit 1 and the scarified tarred skin of Rabbit 3 and Guinea Pig 2 were reinoculated. Some of the material was also inoculated intracerebrally into Rabbit 4 and into the scarified cornea of Rabbit 5. Thus, with fresh material obtained on the 3rd and 4th days of the disease, five rabbits and two guinea pigs were inoculated in various ways.

The animals were carefully observed each day following the inoculations, but in none of the animals were any macroscopic changes seen which could be ascribed to the inoculations. One of the eyes of Rabbit 2 was removed on the 3rd day and the other on the 7th day following the inoculations and sections were made through the corneas. The sections show in places what are apparently the results of mechanical injuries and in the section of the eye removed on the 7th day, foci of slight infiltration of the substantia propria with small round cells. Some swelling of certain of the epithelial cells is also seen. But nowhere are there any signs of vesicle formation or marked inflammatory reaction and no inclusion bodies were found.

Although no definite reactions were obtained in this first series of animals it was thought that by inoculating from one cornea to another and from one brain to another, the virus might possibly become adapted to the rabbit and produce definite lesions in subsequent transfers. Therefore, starting with Rabbit 1 inoculations were made from one rabbit to another by scraping the cornea and washing out the conjunctival sac with a small amount of saline and inoculating the material thus obtained into the scarified cornea of another rabbit. Fourteen corneal passages were thus made, at 2 and 3 day intervals. In many of the rabbits, the scarified eye on the day following the inoculation showed a slight degree of opacity along the lines of scarification and a slight exudation. However, it was found

[2] The eyes of the rabbits were anesthetized locally with cocaine before inoculation. For other operations the animals were given ether.

during the course of the study that slight changes of this character frequently occur following the inoculation of an emulsion of normal rabbit cornea, and even after scarification alone without the injection of any foreign matter whatever. Except for these slight non-specific reactions no changes were observed in any of the eyes of the series. In certain instances, although no gross changes were present, the cornea was sectioned but no lesions which could be considered specific and no intranuclear inclusion bodies were found.

Starting with Rabbit 4 inoculated intracerebrally with the material from this case, ten brain to brain transfers were made at 5 day intervals. Each animal was killed with ether, and the brain removed with sterile precautions. An emulsion of the brain was made with Locke's solution in a sterile mortar, the suspension centrifuged at low speed, and 0.2 cc. of the supernatant fluid injected intracerebrally into a normal animal. At the same time, some of the brain emulsion was inoculated intracorneally and intradermally into each of two other rabbits. It was found that the inoculation of brain emulsion into the scarified cornea usually was followed by conjunctivitis of considerable severity which, however, proved to be wholly non-specific. The temperature of the intracerebrally inoculated rabbits was taken daily, and sections of the brain of each of the inoculated animals made. None of the rabbits showed a significant rise in temperature and careful study of the brain failed to reveal any characteristic lesions. No intranuclear inclusion bodies were found.

Case II.—F. T. Age 13. Patient admitted to the hospital Nov. 13, 1924, suffering from rheumatic fever and cardiac disease. History unimportant in the present connection except that the patient stated that he had had chicken-pox. On the afternoon of Feb. 1, 1925, the patient showed a definite rise in his pulse rate (120). This was attributed to the excitement due to visitors and playing a game. There was no rise in temperature. On Feb. 2 there was discovered a vesicular (herpetiform) eruption over a small area on the lower right chest midway between the nipple and the costal margin. He did not complain of pain or itching and there was no constitutional disturbance. On Feb. 4 when the vesicles were already beginning to become dry, the patient complained of slight itching. A small piece of skin where the rash was present was removed at this time. A portion of this was cut into sections and showed characteristic multilocular vesicles, with swollen epithelial cells containing intranuclear inclusion bodies.

This patient was in the hospital at the same time as Patient I. The two patients were at opposite ends of the same floor, but in different wards. Case II developed nearly 2 months after Case I, and it is not likely that there was any direct connection between them.

The lesion in Case II was so slight that it was thought the condition might be that described in the literature as zosteriform herpes rather than true herpes zoster. The distribution of the vesicles was suggestive of herpes zoster, but the vesicles remained small, the patient never complained of pain in the affected area, and there was no fever.

In view of the clinical course in this case and the somewhat uncertain diagnosis

it was decided to inoculate all the material obtained into the corneas of rabbits. If the lesion were due to herpes simplex virus which had accidentally become implanted on the thorax, the inoculation of the material from this case into the scarified corneas of rabbits should produce typical keratitis. If, on the other hand, the condition were true herpes zoster, it would be advisable to employ as nearly as possible the technique used by Lipschütz. Consequently the piece of skin removed from the affected area was ground up in a mortar without sand in a small quantity of normal saline solution. Portions of the suspension were then rubbed into the scarified right corneas of six young rabbits. Cocaine was used instead of ether to avoid lacrymation. After inoculation the eye was held in position for a short time so that the inoculum might dry on the cornea. The left cornea of each animal was scarified in the same way as the right and left uninoculated to serve as control.

Daily comparisons were made between the inoculated corneas and the corneas which had merely been scarified. In some of the animals a slight conjunctivitis was observed in one or the other eye. But whereas in one instance the reaction seemed more intense in the inoculated eye, in another the control eye appeared worse. In none of the animals did a severe keratitis develop. The possibility that the infective agent in this case might be herpes simplex virus was, therefore, in all probability excluded.

On the 4th day after inoculation although no definite macroscopic lesion developed, the right eyeball of each of the six rabbits was removed, fixed in Zenker's solution, and sectioned. Careful examination of the sections of the six corneas of the inoculated rabbits failed to show any definite lesions or the presence of intranuclear inclusion bodies.

Case III.—J. L. Age 16. The patient was admitted to the hospital Oct. 5, 1924, suffering from diffuse glomerular nephritis. The patient stated that neither he nor any other member of his family had had chicken-pox. On Feb. 11, a vesiculopapular eruption appeared on the lateral and anterior aspects of the left side of the lower chest and upper abdomen, extending over the lumbar region to the midline behind. The eruption occupied the region corresponding to Head's eighth dorsal area and was typically that of herpes zoster. It is of interest that in this patient there appeared on Feb. 17, on the right or opposite side, over the abdomen and flank, a vesicular eruption. The eruption here was exactly like that on the opposite side but much less extensive, and also had a zonal distribution, but slightly lower than on the left, corresponding to Head's tenth dorsal area. Bilateral distribution of the rash in herpes zoster is rare though it does occur, as in this case. This patient occupied a bed directly opposite that of F. T., Case II, and the rash first appeared just 10 days after that of F. T. As herpes zoster is extremely uncommon in this hospital it seems reasonable to suspect some connection between these two cases and also some relation between these cases and Case IV. On the day after the rash appeared a small piece of skin where the rash was present was removed and a little vesicle fluid was obtained. At this time most of the rash was still in the papular stage. A portion of the skin was sectioned and the

examination showed the typical lesions of herpes zoster with many cells containing intranuclear inclusion bodies.

The skin removed on Feb. 12 was ground up in a mortar with Locke's solution and combined with the vesicle fluid. The material was centrifuged at low speed for a few seconds to throw down the larger particles, and about 1.5 cc. fluid was obtained. Monkey 1 (*Macacus rhesus*) was inoculated with this fluid in the following ways: intracerebrally, intracutaneously, into the scarified cornea, and directly into the anterior crural nerve. No reactions occurred at the sites of the corneal and intracutaneous inoculations. No nervous or other symptoms developed. The monkey later developed tuberculosis and was killed on Mar. 9.

On Feb. 14, 1925, the 3rd day of the disease, the "roofs" of four more vesicles were removed, ground up without sand, and inoculated into the scarified corneas of six rabbits. Some of the same material was also inoculated into the scarified skin on the arm of a human volunteer whose history was negative for chicken-pox and herpes zoster. The result of the latter experiment was negative. Three of the inoculated rabbits were bled to death after 24 hours, the eyeballs removed and fixed in Schaudinn's solution and stained with Giemsa. The other three rabbits were observed for 72 hours, the eyeballs removed and treated in the same way. The sections of the corneas of these six rabbits showed no lesions other than those resulting from the scarification. No intranuclear inclusion bodies were found.

Case IV.—R. N. Age 37. This patient developed the lesions of herpes zoster on Feb. 22, 1925. He occupied a bed in the same ward as Patients II and III, and the rash appeared 11 days after that of Case III. The patient was an adult man, 37 years of age. He was admitted to the hospital on Dec. 12, 1924, suffering from diffuse glomerular nephritis. He stated that as far as he knew he had never had chicken-pox. On Feb. 22, 1925, a vesicular eruption appeared over a zone extending over the right lumbar region posteriorly and just below the umbilicus anteriorly. The area of distribution corresponds roughly to Head's tenth dorsal area. The rash was not preceded by any subjective symptoms and was not noticed by the patient until pointed out to him by another patient. The patient seemed quite comfortable on this date. On Feb. 23 the eruption became more marked, and groups of large, tense vesicles were present. Considerable hyperesthesia developed in the involved area. The patient's temperature rose to 101.6° and he complained of general malaise. On the following days he complained of pain, both superficial and deep, along the course of the involved nerve root. The deep pain was of a sharp, cutting character, and paroxysmal in type, and was referred to the right iliac region. Some of the larger vesicles later became hemorrhagic and an enlarged, very tender axillary lymph node appeared on the affected side.

On Feb. 24, the 3rd day of the disease, some vesicle fluid was collected in a sterile syringe containing a trace of sodium oxalate solution. The fluid was injected into the spinal cord of Monkey 2 (*Macacus rhesus*). The monkey was shaved over the dorsal region on both sides so that in case a rash developed it would be

visible. Some of the vesicle fluid clotted before all the injections could be made and the clot was placed in a mortar and ground up with a small amount of salt solution. This material was injected intracerebrally and intracutaneously into Monkey 2, and a small amount was also rubbed into the scarified cornea. The results of the skin and eye inoculations were completely negative. No cutaneous reactions appeared at the level of the spinal cord inoculation. The monkey never showed any nervous or other symptoms. No rise in temperature was observed.

On Mar. 28, a month after the inoculation with herpes zoster material, the monkey's susceptibility to herpes simplex virus was tested by intracerebral inoculation of a virulent herpes simplex virus. The monkey never showed a febrile reaction or any other symptoms.

On Feb. 24 vesicle fluid from the patient was obtained and immediately rubbed into the scarified cornea of a rabbit. The rabbit showed a slight amount of conjunctivitis after 24 and 48 hours, but the eye appeared normal after 72 hours.

On Feb. 28 the "roofs" of four vesicles were removed. This tissue was ground without sand, mixed with a small amount of saline, and the suspension was injected intracranially into two rabbits and intraspinally into two others. The rabbits which received the injections into the cord were shaved at the level of inoculation so that any skin reaction would be visible. All four of the rabbits remained well, and never showed any febrile or other reaction.

Case V.—E. K. Age 19. Student. The patient was admitted to the hospital May 23, 1925, with the diagnosis of herpes zoster. The past history is unimportant except that he is sure that he had chicken-pox at the age of 5. On May 18, 5 days before admission, he first noticed a slight dull pain in his chest on the left side, and this pain persisted. On May 20 the pain became much worse, and an eruption of "small blisters" over the left breast, anterior to the axilla, was noted. He had slight fever and slight general malaise. He described the pain as being a "stinging itch;" sometimes it became a dull, constant pain. The pain was present not only over the area involved in the rash, but radiated to the lower left back. On admission there was present on the left side of the thorax an irregularly arranged, rather sharply delimited, papulovesicular eruption. The eruption occupied, roughly, a horizontal zone 6 to 8 cm. broad, just below the level of the nipple, occupying Head's fifth dorsal area. The vesicles varied in size, and were mostly discrete. There was slight tenderness over the affected area and there was some hyperesthesia of the uninvolved skin in this area. A small piece of the affected skin was removed on May 23. Microscopical sections of this showed typical vesicles of herpes zoster with numerous intranuclear inclusion bodies. At the same time three other small pieces of skin were obtained and ground without sand in a sterile mortar in a small amount of Locke's solution. 0.2 cc. of the supernatant fluid of this emulsion was injected into each testicle of a young vervet monkey (*Cercopithecus lalandii*), commonly called the green monkey. The monkey's temperature remained normal on the succeeding days. On the 4th day after inoculation the left testicle was removed. No gross changes were evident. The whole testicle was placed in Zenker's solution and the following day was divided

into five blocks and sections were cut from each block. On the 5th day after inoculation the right testicle was removed and treated like the left. Numerous sections from both testicles were studied and, although the left testicle seemed to show a definite lesion with hemorrhage and necrosis, no typical intranuclear inclusion bodies were found in sections from either testicle.

The attempt to inoculate a vervet monkey with material from this case was, therefore, unsuccessful. There is still disagreement among the various investigators as to whether monkeys are susceptible to herpes simplex virus. An attempt was made to infect this monkey later with fresh virulent herpes simplex virus but no reactions occurred. This species of monkey is therefore probably insusceptible or at least not highly susceptible to herpes simplex virus.

Case VI.—P. C. Age 41. The patient was admitted June 30, 1925. He was uncertain whether or not he had had chicken-pox. On the evening of June 25, 1925, when the patient was walking home from work he felt an intense itching sensation around the right scapular region, extending anteriorly around into the right axillary region. Later in the evening small vesicles began to appear in this area. The rash appeared over the right scapula and anteriorly below the right mammary region, the distribution corresponding to Head's third and fourth dorsal areas. There was considerable pain in the involved regions.

On June 30, on the 6th day of the disease, four small pieces of skin were removed and ground in a sterile mortar without sand in a small amount of Locke's solution. 0.1 cc. of this emulsion was injected into each testicle of a young vervet monkey. On the following day, July 1, 1925, the monkey's temperature showed no striking rise. Both testicles were enlarged. 24 hours after inoculation the left testicle was removed. The whole testicle was placed in Zenker's solution, and the next day was divided into three blocks. July 2, the right testicle was removed and treated in the same way. Numerous sections from both testicles were studied, but no specific lesions with intranuclear inclusion bodies were found.

Case VII.—J. S. Age 38. The patient was admitted July 2, 1925. The patient has syphilis and had received antisyphilitic treatment at the Presbyterian Hospital since March. She did not know whether or not she had had chicken-pox. For 2 or 3 days before admission she had pain over her left breast and over the left side of her back in the scapular region and in her left arm. On July 1, small vesicles appeared on her back and on the left breast, and a fairly large crop of vesicles appeared in the axillary region. The distribution corresponded to Head's fourth dorsal area.

On July 2, 1925, the 2nd day of the disease, some vesicle fluid was collected from the larger vesicles, and seven small pieces of skin were removed and ground up without sand in a small amount of Locke's solution. The vesicle fluid was added to the skin emulsion, and 0.2 cc. of this material was injected into each testicle of a young vervet monkey. An intracutaneous injection was also made. The following day the testicles appeared enlarged. 40 hours after inoculation the right testicle was removed and sections made. 72 hours after inoculation the left testicle was removed and sections made. Numerous sections of both

testicles were studied, but no specific lesions with intranuclear inclusion bodies were found. The result of the intracutaneous inoculation was completely negative.

Inasmuch as this patient was receiving injections of salvarsan and mercury, it was thought that this case, in spite of the typical clinical course and characteristic distribution of the vesicles, might represent an example of so called symptomatic herpes zoster described particularly by Cipolla and in the cases of which he found the virus of herpes simplex. Tests were therefore made for the presence of herpes simplex virus by inoculating some of the material from this case into the eyes of four rabbits, but the results were negative.

Case VIII.—S. B. Age 14. The patient was admitted July 13, 1925. The patient had been in the hospital since May 22 suffering from pleurisy. She stated definitely that she remembered having had chicken-pox at the age of 6 or 7 years. On July 12 she complained of pain in her right shoulder and on examination a vesicular rash covering the right scapular region was found. The eruption extended from the lower half of the wing of the scapula to the vertebral column. A few isolated vesicles were present anteriorly to the right of the nipple. The area involved corresponded to Head's fourth dorsal segment. A slight hyperesthesia of the skin was noted.

On July 14, the 3rd day of the disease, three fairly large pieces of the involved skin were obtained and ground up without sand in a small amount of Locke's solution. Injections of this material were made in the following ways. It was injected into the testicles of a young vervet monkey, intracutaneously into the scarified skin of one tarred guinea pig and one tarred rabbit, and into the scarified corneas of two rabbits. The inoculated eyes and skin remained completely negative. On July 15 the monkey's temperature was 103°. On July 16 the temperature was 100.6° and the right testicle was removed for section. On July 17 the left testicle was removed. Numerous sections of both testicles were studied but no specific lesions with intracellular inclusion bodies were found.

Case IX.—H. S. Adult male. The patient had had chicken-pox in childhood but had never had herpes zoster and had not recently been in contact with any case of herpes zoster. His wife had had a herpes simplex eruption a short time previously. The papular rash over the right shoulder appeared after 2 weeks of slight indisposition and after several days of severe headache and pain in the back.

On the 1st day of the eruption the tops of three papules were removed, immediately ground without sand, and injected into both testicles of a young vervet monkey. There occurred no febrile reaction and on the 3rd day the left testicle was removed and on the 4th day the right testicle was removed. Evidences of injury with hemorrhage were seen but on section no marked cellular reactions were present and no inclusion bodies were found. Some of the material from this case was also inoculated into the scarified corneas of two rabbits with negative results.

SUMMARY AND DISCUSSION.

Attempts were made to produce lesions in animals by the injection of material obtained from the vesicles and involved skin of nine cases of herpes zoster. All the cases, with the exception of one (Case II), were characteristic cases of idiopathic herpes zoster and the question of their being cases of so called zosteriform herpes or symptomatic herpes zoster can hardly be raised. As regards Case II, if this case occurred alone, there might be some doubt as to its nature on account of the mildness of the symptoms and the small area of skin involvement. Taken in connection with Cases III and IV, however, which occurred in the same ward and in patients who were quite closely in contact with Patient II, it seems fairly reasonable to assume that they were all of the same character. Cases of herpes zoster have been extremely rare in this hospital and the occurrence of three cases in the same ward within a very short period of time suggests very strongly a transference of infection from one case to the other. That Case II was not one of herpes simplex also seems fairly certain from the negative results obtained by inoculation of rabbits' eyes with vesicle material.

In making the animal experiments we employed various methods which were suggested largely by the technique used by previous observers, especially by those who have reported results which were considered positive. In making inoculations into the corneas the technique recommended by Lipschütz was employed as far as possible. Young rabbits were used and the material was obtained from fresh vesicles early in the disease and inoculated with as little delay as possible. The material injected into rabbits' eyes was obtained from seven cases and twenty-four rabbits were used. In judging of the results obtained in this kind of experimentation great caution must be observed. Our experience convinces us that slight opacities occurring along the lines of scarification and mild conjunctivitis cannot be held to indicate the effect of a specific virus. As regards the interpretation of the microscopic changes found, we were quite familiar with the appearance of intranuclear inclusion bodies as seen in the lesions of experimental herpes simplex and the filterable virus (Virus III) indigenous to rabbits described by Rivers and Tillett (5). We

also had no difficulty in finding intranuclear inclusions in the sections of skin removed from patients. It is not likely, therefore, that these structures were overlooked in our study of the sections. Briefly stated, although the material studied was satisfactory and in spite of the fact that a considerable number of animals were used for each case, we have been unable to confirm the observations of Lipschütz regarding the experimental production of specific lesions in the corneas of rabbits. We realize that this is only negative evidence and therefore not of conclusive importance in view of Lipschütz's observations. It indicates, however, that the production of specific lesions in rabbits' eyes with material from herpes zoster vesicles is extremely difficult and that successful results may be a matter of chance, depending, possibly, on peculiar susceptibility on the part of the rabbits. In view of the fact, however, that a careful analysis of the positive results reported by other observers shows that the conclusions were based on insufficient evidence, we believe that further work is necessary before the successful inoculation of the rabbits' corneas with herpes zoster virus can be accepted as fully demonstrated. To make the evidence convincing specific lesions should be obtained with a fair degree of regularity and the virus should be successfully transmitted through at least two generations. Apparently the latter was not attempted by Lipschütz.

Intracerebral inoculations into three rabbits with material from two cases (Nos. I and IV) were made. Two rabbits were also inoculated intraspinally with material from one case (No. IV). None of these animals showed any reaction. In the case of one of the animals inoculated into the brain (Case I) although this rabbit showed no symptoms, we thought it conceivable that the susceptibility of the species for the virus might be so slight that no obvious lesion had been produced. Nevertheless it was thought that the virus might possibly remain alive at the seat of inoculation and by repeated transfers become adapted to the rabbit. This phenomenon has been observed by Noguchi with vaccine virus, and by Rivers and Tillett with the rabbit virus isolated by these workers. This possibility was tested by us by making serial corneal and brain inoculations. Corneal transfers were carried through fourteen animals in series, and brain transfers through ten. No specific lesions developed in any of the animals.

The work of Teague and Goodpasture suggested that the skin might be rendered more susceptible to infection by previous treatment with tar. Material from two cases (Nos. I and VIII) was inoculated into the tarred skin of guinea pigs and rabbits. The material was injected intracutaneously and also rubbed into the scarified skin. No reaction was obtained in any of the animals.

Finally, the transmission of herpes zoster to monkeys was attempted. Blanc and Caminopetros, and Bastai and Busacca, as discussed in the review of the literature, inoculated monkeys (*Macacus*) in various ways, without success.

It was thought possible that although monkeys of the genus *Macacus* might be refractory, monkeys of another genus might prove susceptible. Consequently, besides the inoculation of two *Macacus* monkeys, attempts were made to infect five vervets. Moreover, in view of the fact that the virus of vaccinia and the rabbit virus of Rivers and Tillett could be successfully cultivated in the testicle, intratesticular inoculations were employed. The testicles were removed at varying periods following inoculation. Numerous sections of these testicles were made and examined, but in no instance were any lesions found which could be interpreted as specific. No cells containing intranuclear inclusion bodies were found. These experiments, therefore, have also led to purely negative results.

This report of our work is made at the present time because a considerable amount of literature has been published which gives the impression that herpes zoster has been successfully transmitted to animals. Although the observations of Lipschütz are suggestive, it is important that they be confirmed by further investigations.

Until herpes zoster can be regularly transmitted to animals and cross-immunity tests be carried out, the relation of the virus of herpes zoster to that of herpes simplex remains a matter of speculation. In view of the fact that herpes simplex can be easily and regularly transmitted to rabbits, whereas in the hands of a large number of investigators similar experiments with herpes zoster are completely negative, it does not seem likely that the etiological agent concerned in these two diseases can be absolutely identical.

The question of the identity or non-identity of herpes zoster and varicella is even more difficult to answer, because at present neither

of these infections is readily transmissible to animals. The work of Kundratitz is extremely interesting. His observations, aside from indicating a close immunological relationship between herpes zoster and varicella, are important in that they seem to show the presence of a transmissible virus in the vesicles of herpes zoster. The only question that arises is whether the cases of herpes zoster from which Kundratitz was able to make successful transfers were true cases of idiopathic herpes zoster.

CONCLUSION.

Attempts to inoculate rabbits, guinea pigs, and monkeys with material obtained from nine cases of herpes zoster have proved unsuccessful.

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STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

IV. Concerning the Oneness of the Bacteriophage.

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(Received for publication, August 4, 1925.)

D'Hérelle believes that the bacteriophage is a filterable, ultramicroscopic virus, parasitizing upon and destroying actively growing, susceptible bacteria. According to his conception, "there is but a single bacteriophage, common to both man and animals, capable by adaptation of acquiring a virulence toward all bacterial species."[1]

Evidence exists against this view. Some investigators, having failed in their attempts at adaptation of the bacteriophage,[2,3] believe in the existence of a number of serologically distinct strains, even among closely related varieties of the lytic principle.[4–8] This conception finds support in the fact that the secondary overgrowth of bacteria, appearing in the culture in the presence of lytic principle, is specifically resistant to lysis by the agent conditioning its appearance, but is often susceptible to the lytic action of another closely related lytic filtrate.[2, 5, 9, 10] Further evidence in favor of a multiplicity and relative specificity of lytic principles is to be seen in the differences in the characteristic size and appearance of plaques, or sterile spots,

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when bacteria are grown in the presence of different lytic filtrates on the surface of agar.[10, 11]

In the course of experiments preliminary to a study of the influence of hydrogen ion concentration on the adsorption of lytic principle by bacteria, we have made observations which, in our opinion, are favorable to this view of a multiplicity of the lytic principle. It was found that the four lytic filtrates which were examined showed distinct differences in the response to changes in the hydrogen ion concentration of the medium, irrespective of the species of bacteria serving as substratum for the production of the active filtrates.

The experimental procedure was as follows:

Influence of Temperature and of Duration of Exposure.

Lytic filtrates, prepared by growing susceptible bacteria in the presence of active principle upon unbuffered broth, adjusted to pH = 7.4, were diluted 1:1000 in physiological salt solution,[12] and 0.2 cc. of this dilution was added to a series of tubes, each containing 1.8 cc. of buffer solutions, varying from pH = 2 to pH = 12 (thus diluting the original filtrate 1:10,000). These mixtures were kept at a constant temperature, and after a stated period of time 0.1 cc. of the contents of each tube of the series (containing now 10^{-5} cc. of the original filtrate) was transferred to 9.9 cc. of sterile buffered broth (pH = 7.4), and titrated by the serial dilution method.[13, 14]

Electrometric measurements have shown that the addition of 0.2 cc. of diluted filtrates to 1.8 cc. of the various buffers did not change appreciably their respective hydrogen ion concentrations. Similarly it was established that the subsequent transfer of 0.1 cc. of the mixture of various buffer solutions containing the lytic principle into 9.9 cc. of buffered broth (pH = 7.4) resulted, to all intents and purposes, in the immediate neutralization of solutions added.[15]

In the study of the adsorption of lytic principle by bacteria, to which this series of experiments was a preliminary, the tests were carried out at a temperature of +7°C., in order to avoid the rapid multiplication of bacteria and a corresponding increase in the concentration of the lytic principle during incubation. All the experiments reported in the present paper were performed at this tem-

[11] Watanabe, T., *Z. Immunitätsforsch., Orig.*, 1923, xxxvii, 106.

[12] Dilution was resorted to in order to shorten the subsequent titration series.

[13] Appelmans, R., *Compt. rend. Soc. biol.*, 1921, lxxxv, 1098.

[14] Werthemann, A., *Arch. Hyg.*, 1922, xci, 255.

[15] In the case of the extremely acid (pH = 2) and extremely alkaline (pH = 12) mixtures, the addition of 0.1 cc. of these mixtures to the 9.9 cc. of broth caused a drop or rise of the pH of the medium of less than 0.2.

perature (+7°C.). However, we carried out one of the experiments at both +7°C. and 37°C., in order to ascertain the effect of higher temperature on the rate of deterioration of the lytic principle at different pH. In the experiment mentioned lytic filtrate was allowed to remain in contact with various buffers, at 7°C., and at 37°C., for 3, 6, and 24 hours respectively. At the end of each period a sample (0.1 cc.) was taken from each mixture and titrated by serial dilution.[13, 14] The results of the titrations were first read at the end of 24 hours incubation at 37°C.; then again at the end of a further period of 24 hours of incubation. At this time the lytic titer in each of the tubes which received originally one or more active lytic units would have reached its maximum. For this reason, the tubes in which the occurrence of lysis was doubtful were heated for 30 minutes at 56°C., in order to destroy the resistant bacteria, and now 0.1 cc. of the contents of such tubes was transferred into a fresh tube containing 10 cc. of sterile broth, seeded with 0.1 cc. of an emulsion containing 1,000,000,000 susceptible bacteria per cc., and incubated for 3 to 4 hours at 37°C. At the end of this time, and before any overgrowth had taken place, the final readings were made.

As an illustration, results of the experiment at 7°C. are shown in Protocol 1. The sign + in this protocol signifies the presence of lysis, as judged by absence or slight amount of visible growth, and the sign − signifies apparent absence of lysis, or presence of bacterial growth equal or nearly equal to that occurring in a control tube of broth receiving 0.1 cc. of bacterial suspension only.

It will be seen from this protocol that direct readings of the results of titration, after 24 or 48 hours, were somewhat confusing, owing to the fact that the overgrowth of resistant bacteria often masks the coincident lysis of susceptible individuals in the culture. It is for this reason that a final test, by means of a transfer of 0.1 cc. from each tube (after heating) was resorted to, as stated above.

The results recorded in the protocol represent quantitatively the changes in activity of the Laudman Shiga lytic filtrate, which took place after its exposure to various buffers for 3 hours at + 7°C. Identical titrations were performed after a period of 6 and 24 hours contact of filtrate with buffers at 7°C., as well as after a contact for the same periods of time at 37°C. The results of all these titrations are presented graphically on Chart 1, in terms of the minimum amount of the original mixture (Protocol 1, *A*) which contained at least one active unit of the lytic principle after the exposure to buffers for a fixed length of time, at a fixed temperature.

Protocol 1.

Effect of the Hydrogen Ion Concentration on Activity of the Lytic Filtrate Laudman Shiga at +7°C.

A	Laudman Shiga filtrate diluted 1:1000, *cc.*	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	
	Buffer solutions, *cc.*	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	
	pH of buffer solutions (approximate)	2.0	3.0	4.0	5.0	6.0	7.4	8.6	9.8	11.0	12.0	
	Resulting pH of mixture (electrometric)	2.05	3.1	4.1	5.1	6.15	7.4	8.77	9.75	11.25	12.07	

Mixed. Left in ice box. Samples taken after 3 hrs.

	Amount of sample, *cc.*	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0
	Buffered (pH = 7.4) broth, *cc.*	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9	10.0
	Resulting pH (electrometric)	7.25					7.4				7.6	7.4

1 cc. of the contents of each tube, after thorough mixing, transferred into 9.0 cc. of broth, and 1 cc. from that tube into 9.0 cc. of broth in the next series, and so on.

	Absolute amount of the original lytic filtrate present.	Amount of Mixture A present.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.
	cc.	*cc.*																																				
Results of titration after 3 hrs.	1×10^{-5}	0.1	−	−	−	−	−	−	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	+	+	+	−	−	−			
	1×10^{-6}	0.01	−	−	−	−	−	−	+	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	−	−	−	−			
	1×10^{-7}	0.001	−	−	−	−	−	−	+	±	+	±	+	+	±	−	+	−	−	+	−	−	+	+	−	−	+	−	−	−	−	−	−	−	−			
	1×10^{-8}	0.0001	−	−	−	−	−	−	+	+	+	+	+	+	+	−	+	±	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−			
	1×10^{-9}	0.00001	−	−	−	−	−	−	+	+	+	+	+	+	+	−	+	−	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−			
	1×10^{-10}	0.000001	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+ *	−	−	−	−	−	−	−	−			
	1×10^{-11}	0.0000001	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−			
	Control.	0.0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

* Occasional activity in this dilution was due to particulate nature of distribution of the active principle (see earlier paper: Bronfenbrenner, J. J., and Korb, C., *J. Exp. Med.*, 1925, xlii, 483).

Comparative Resistance to Changes in Hydrogen Ion Concentration.

As can be seen from the chart, the destruction of the lytic power of the filtrate is considerably greater at 37°C. than at 7°C. So far as the effect of the length of exposure is concerned, in either case practically the entire change takes place very early, so that very little fur-

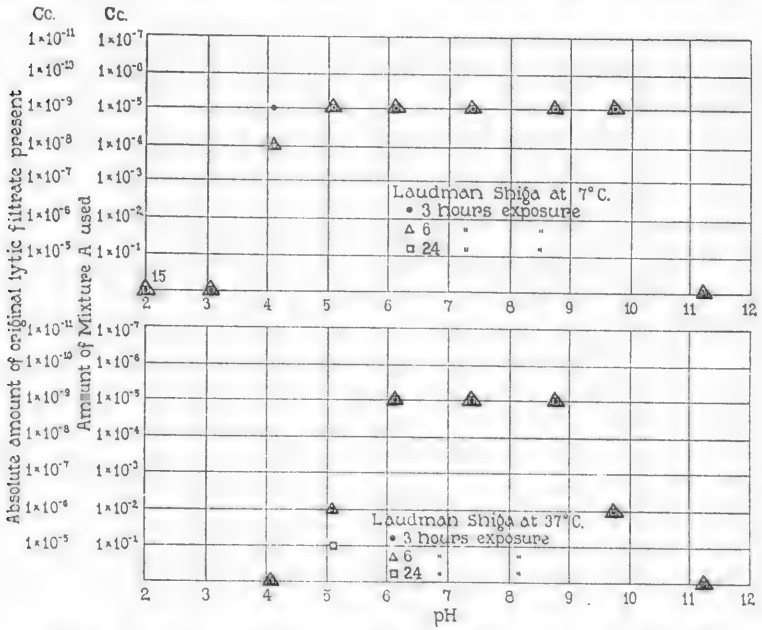

CHART 1. Effect of changes in the hydrogen ion concentration on the activity of lytic filtrate. On this as well as on the two other charts the points plotted on the base line indicate that there was no lysis in the largest amounts used (1×10^{-1}).

ther deterioration can be noted after the lapse of the first 3 hours. For the reasons already stated, in all further experiments the exposure of lytic filtrates to different buffers was carried out at 7°C. only, and, because of the above findings, the exposure in all cases was limited to 3 hours.

Using the procedure described, we have studied the resistance to changes in the hydrogen ion concentration of lytic filtrates active

respectively against *Bacillus coli*, *Bacillus dysenteriæ* Shiga, *Bacillus pestis caviæ*, and staphylococcus. Since the protocols of these experiments are in every detail similar to Protocol 1, further protocols have been omitted, and the findings have been recorded in Chart 2, in the manner identical with that used for construction of Chart 1.

Effect of "Adaptation" on the Characteristic Resistances.

The data plotted on Chart 2 have been obtained repeatedly, thus indicating that the various lytic agents studied have individual curves

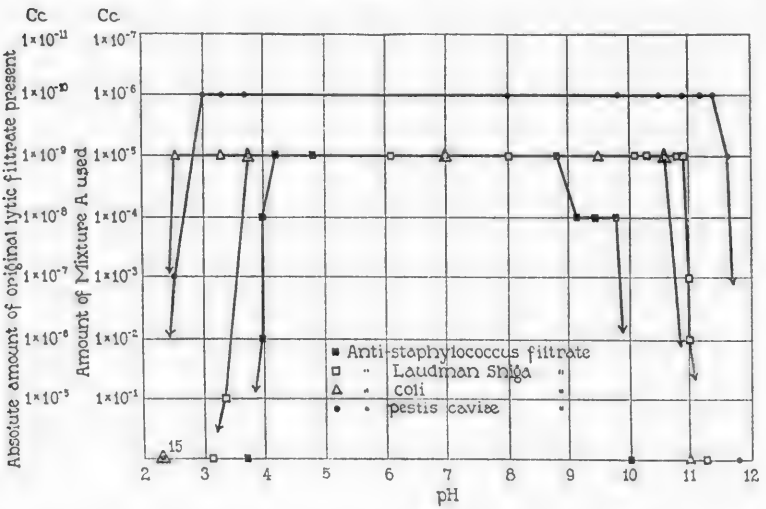

CHART 2. Comparative resistance of different lytic filtrates to changes in hydrogen ion concentration.

of resistance to changes in hydrogen ion concentration. The lytic agents active against *Bacillus pestis caviæ* seem to be the most resistant, remaining unaffected in the wide zone between pH = 3 and pH = 11.45. The lytic agent active against staphylococcus, on the other hand, is the least resistant, its resistance being limited by the hydrogen ion concentration corresponding to pH = 4.2 on the acid side, and pH = 8.8 on the alkaline side. The lytic filtrates active against *Bacillus coli* and *Bacillus dysenteriæ* Shiga resist alkalinity in almost the same degree, the limiting alkalinity for the former being pH = 10.6 and for

the latter pH = 10.9. On the acid side the lytic agent active against *Bacillus coli* is limited by pH = 2.55, whereas that active against *Bacillus dysenteriæ* Shiga deteriorates beyond pH = 3.75.

If one accepts d'Hérelle's conception that there is only one lytic principle these differences would have to be explained as resulting from "adaptation" of the virus to different "hosts."

In order to determine whether the change in bacterial species serving as substratum for the production of lytic filtrate is sufficient to cause the change in its resistance to acidity or alkalinity, the following experiment was undertaken.

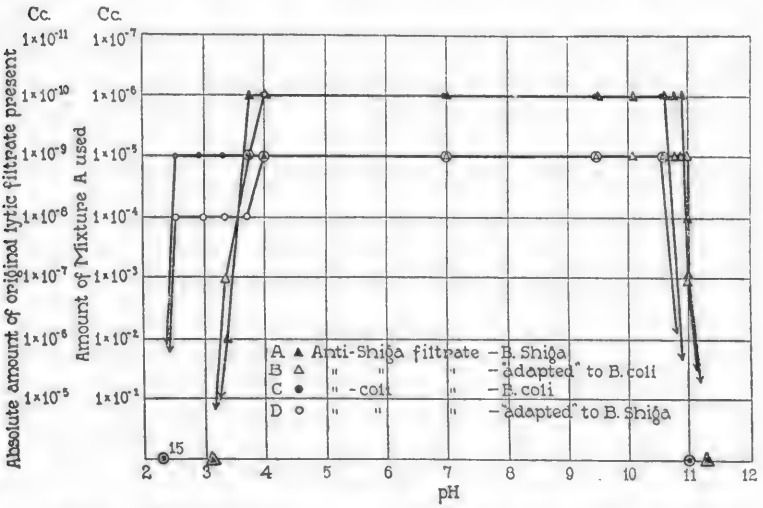
Cc.
Cc.
Absolute amount of original lytic filtrate present
Amount of Mixture A used
A ▲ Anti-Shiga filtrate – B. Shiga
B △ " " " – "adapted" to B. coli
C ● " - coli " – B. coli
D ○ " " " – "adapted" to B. Shiga
15
pH

CHART 3. Effect of "adaptation" on resistance of lytic filtrates to changes in hydrogen ion concentration.

Starting with a polyvalent lytic principle, acting primarily against *B. dysenteriæ* Shiga and secondarily against *B. coli*, two lytic filtrates were prepared; one (*A*), by passage through a number of generations upon a culture of *B. dysenteriæ* Shiga, and the other (*B*) upon a culture of *B. coli*. The number of passages (ten) was amply sufficient to have eliminated all traces of activity due to the original filtrate. At the end of these passages the filtrates thus obtained caused lysis of the respective bacteria when present in culture in the amounts of 10^{-10} and 10^{-9} cc. respectively.

In a similar manner two filtrates were prepared, starting with another polyvalent

bacteriophage, active principally against *B. coli*, though causing also a less complete lysis of Shiga bacillus. In this case one filtrate represented the result of many passages on a culture of *B. coli* (*C*), and the other ten passages on Shiga bacillus (*D*).

All four filtrates were diluted with buffer solutions at various pH and titrated after exposure to them for 3 hours at 7°C., exactly as before. The results of these titrations have been plotted on Chart 3.

Although the initial titers of both the filtrates of the "adapted" bacteriophages (*B* and *D*) were somewhat lower than those of the respective original ones (*A* and *C*), nevertheless the resistance to changes in hydrogen ion concentration was identical for each original and its adapted material.

SUMMARY AND CONCLUSIONS.

Lytic filtrates, active against *Bacillus dysenteriæ* Shiga, *Bacillus coli*, *Bacillus pestis caviæ*, and staphylococcus respectively, proved to be differently affected by changes in hydrogen ion concentration.

Anti-staphylococcus lysin was the least resistant of the four, showing deterioration in 3 hours at 7°C. beyond the zone of hydrogen ion concentration limited by $C_H = 6.3 \times 10^{-5}$ and $C_H = 1.6 \times 10^{-9}$. Under the same conditions, the zone of resistance of anti-*coli* filtrate lay between $C_H = 2.7 \times 10^{-3}$ and $C_H = 2.5 \times 10^{-11}$, and that of anti-Shiga between $C_H = 1.7 \times 10^{-4}$ and $C_H = 1.3 \times 10^{-11}$. Anti-*pestis caviæ* filtrate was most resistant of the four, retaining its full activity in the zone from $C_H = 1 \times 10^{-3}$ to $C_H = 3.5 \times 10^{-12}$.

The fact that these differences in individual resistance persisted, notwithstanding the repeated passage of lytic filtrates through cultures of bacteria other than those against which they were primarily active, seems to offer evidence in favor of a multiplicity of bacteriophages.

STUDIES ON THE RELATION BETWEEN TUMOR SUSCEPTIBILITY AND HEREDITY.

II. The Incidence of Tar Tumors in Strains of Mice Having a Differing Incidence of Spontaneous Growths.

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(Received for publication, July 18, 1925.)

The ability to produce malignant new growths at will in laboratory animals has provided a new and valuable instrument for the investigation of cancer. Fibiger's[1] success in causing neoplasms in the stomach of rats infected with Spiroptera was followed shortly by Yamagiwa and Ichikawa's work[2] with coal tar upon rabbits; and the application of the coal tar method (Tsutsui[3]) to mice, the species in which transplanted and spontaneous tumors are best known, has opened the way to one of the most popular fields of present day medical research. The progress of the growths, the histological findings, the conditions of occurrence and so forth, have been treated in many papers. In all the published accounts it may be noted that there were wide variations in the response given by the animals subjected to treatment.

It is difficult to compare results from different laboratories. Nevertheless the reports indicate that the same tissue in different species shows different degrees of reaction to the treatment designed to elicit neoplasms. The skin of rabbits and mice responds rather readily to irritation with tar, while that of rats and guinea pigs appears to be highly resistant. Not only are species differences indicated but among individuals of the same species a wide range of variability is seen. Apparently no tests have been made heretofore to determine whether

[1] Fibiger, J., *Berl. klin. Woch.*, 1913, l, 289; *Compt. rend. Soc. biol.*, 1920, lxxxiii, 1160.

[2] Yamagiwa, K., and Ichikawa, K., *Mitt. med. Fakult. k. Univ. Tokyo*, 1915–16, xv, 295; 1917–18, xix, 483; *J. Cancer Research*, 1918, iii, 1.

[3] Tsutsui, H., *Gann., Japan Z. Krebsforsch.*, 1918–19, xii, 17.

the individuals manifesting distinct differences may not come from families which have peculiar and characteristic grades of susceptibility.

For some years there have been reported strains of mice which show differences, apparently hereditary, in the rate of incidence of spontaneous tumors, notably those of the mammary gland. Two such strains bred at The Rockefeller Institute seem to afford suitable material for tests as to whether there are hereditary differences affecting the response to treatment with coal tar.

EXPERIMENTAL.

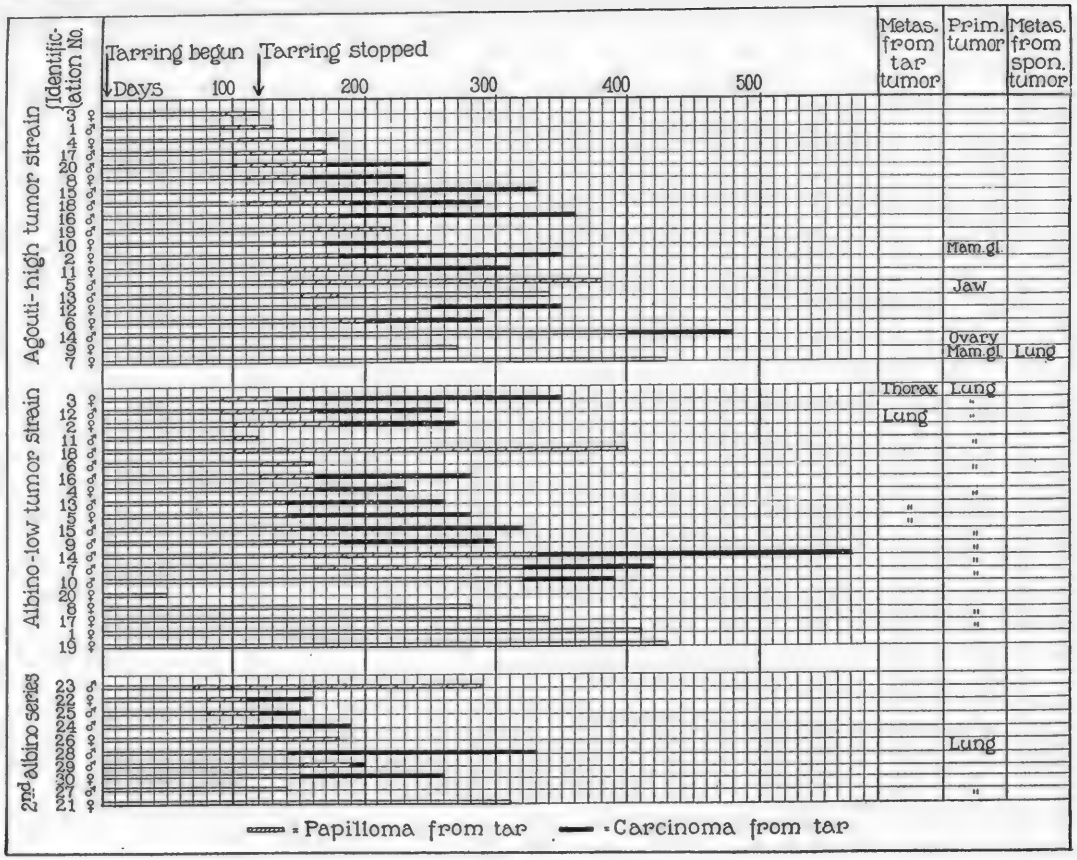
The stocks chosen were a strain showing a high tumor incidence, No. 1194 from the Lathrop mice, and a strain exhibiting a lesser incidence, which was originally obtained from Dr. Bagg at the Memorial Hospital. Strain 1194 comprises strong healthy mice, rather large and of the wild, or agouti, color. Of the bred females reaching the age of 6 months or more, 65 per cent develop mammary gland tumors while those not bred develop them in 20 per cent. The Bagg mice are albino but are also a vigorous stock, in which the animals producing young have a 28 per cent tumor rate and those not bred only 4 per cent. The average tumor age of Strain 1194 is 12 months and of the Bagg mice 18 months.

The technique used was to paint the mice three times weekly for 4 months on a spot between the shoulders, applying approximately 1 drop from an applicator dipped in a solution made from coal tar residue. Depilation was found unnecessary but the hair was clipped before the first painting.

The tar solution used was a benzene extract free from acids, bases, and phenols made from the residue from a coke oven in which the tar had been distilled at a temperature of approximately 377°C. The residue was obtained from the Barrett Company who have very kindly cooperated with us a number of times by providing both residue and extract. We wish to extend our thanks to them.[4]

For the experiment 20 mice from each strain were selected. Approximately half were males and half were females that had not been bred. Tar was applied for 4 months, from the middle of November to the middle of March. The first painting was usually followed by a loss of hair from the treated area. Desquamation could be observed sometimes after a few paintings but the first papillomatous elevation was not noted until after the 90th day. The elevations were strongly keratinizing, often producing horns of remarkable size similar to those pictured by other investigators. When carcinomatous changes occured they became evident about the 140th day or later, either as an induration at the base of the horn or, if the latter had not persisted, at its former site. Occasionally they appeared without the formation of a papilloma. Experience has shown that the induration

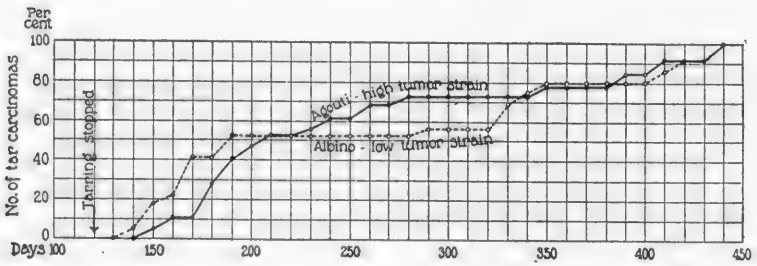
[4] For a more detailed account of the preparation of the tar product see Murphy, Jas. B., and Landsteiner, K., *J. Exp. Med.*, 1925, xli, 807.


Identification No.
Tarring begun
Tarring stopped
Days
100
200
300
400
500
Metas. from tar tumor
Prim. tumor
Metas. from spon. tumor
Agouti-high tumor strain
Albino-low tumor strain
2nd albino series
Mam.gl.
Jaw
Ovary
Mam.gl.
Lung
Thorax
Lung
Lung
= Papilloma from tar
= Carcinoma from tar

TEXT-FIG. 1.

referred to is indicative of carcinomatous transformation of the cells but the diagnosis was confirmed by sections taken at the time of death.

In general the results of the experiment show that no significant difference in the response to tar painting was given by the two strains of mice with differing rates of incidence of spontaneous tumors. Of the 20 mice from the strain with high incidence, all of which survived the period of painting (120 days) 12 eventually developed carcinomas of the skin (60 per cent); from the low tumor strain, the albinos, 19 individuals lived 120 days and again 12 produced carcinomas, approximately the same percentage. Since the mice lived differing lengths of time, it is important to analyze the results more closely. The course of the experiments may be followed in the graphs. The percentages



TEXT-FIG. 2.

from which the graphs were made, are based upon small numbers of individuals and therefore cannot be significant in detail but they are of value in indicating the general progress of events.

The histories of the individuals presented in the first two sections of Text-fig. 1 are condensed and compared in Text-fig. 2. It will be seen that the carcinomatous transformations occurred slightly earlier in the albinos or low tumor strain than in the agoutis. By the 140th day 1 of the 18 mice living at that period showed distinct induration while none of the high tumor strain did. For a time the albinos continued to yield a larger number of individuals with carcinoma, showing at the 170th day 7 out of 17, or 41 per cent, as against 2 out of 18 or 11 per cent among the agoutis; but by the 210th day the percentages were even (9 out of 17 or 53 per cent in each case). From the 190th

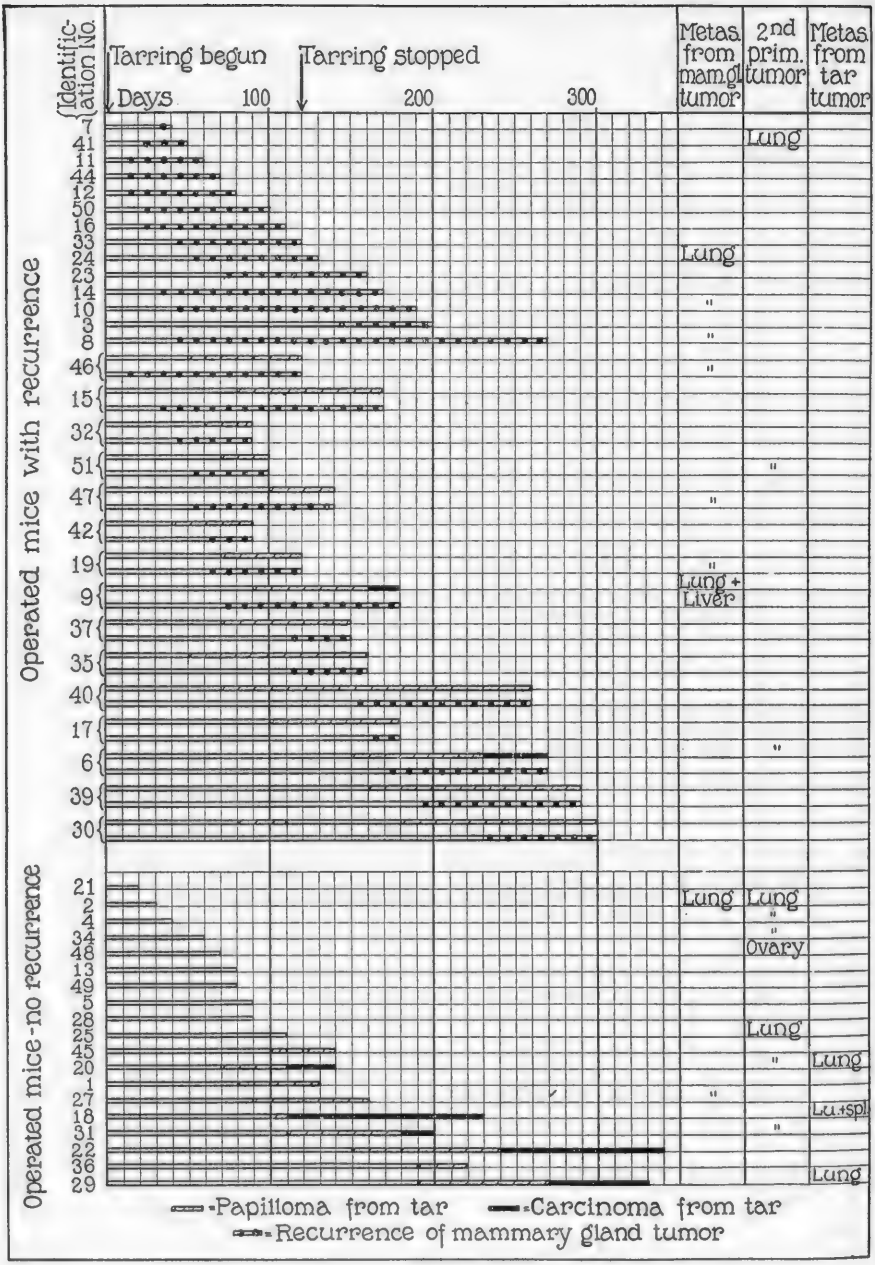
day the percentages are figured from the total number of carcinomas which had appeared in the group and the total number of animals living, or already dead, as a result of the neoplasm. From the 230th to 330th day the tumor rates of the agoutis exceeded that of the albinos but from the latter time to the end of the observations the rates were practically parallel. Three mice of the high tumor strain and five of the low exhibited a high degree of resistance for a considerable period but by the 440th day the sole survivor in each group had a cancer, but of very recent origin in one case.

We see then, that there was a gradual increase in the incidence of tar cancer in both strains, beginning with about the 150th day. The slight variations shown are not regarded as significant. It is to be noted in relation to the period of tarring that papillomas sometimes occur during the 4th month although they may not appear until after the applications have ceased. In the experiment now under consideration the carcinomatous changes took place only after the tarring was discontinued.

The series described were tarred during the winter months. In April an additional group of 10 albinos from the low tumor strain was subjected to the treatment. A few of these animals developed papillomas earlier (80th day) than either the agoutis or the first albinos and induration also was detected sooner in certain cases—in 2 of them before the end of the tarring (Text-fig. 1). Derom[5] has conducted experiments from which he concluded that the temperature of the tar and heating the tissues play a rôle in susceptibility to tar painting. The observations here reported are too meagre to be the basis of definite conclusions in regard to seasonal variations but they are not contradictory to Derom's point of view. Certainly they show the variability which may occur within a given strain and the inadvisability of using so called standardized controls.

The second albino group just mentioned was studied as an additional control for another series of mice. Those previously dealt with had been individuals from strains which produced tumors but which had not yet grown them themselves. It seemed pertinent to enquire

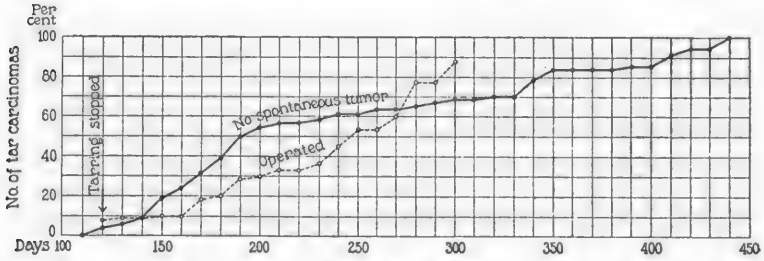
[5] Derom, E., *Bull. Assn. franç. étude cancer*, 1924, xiii, 422.


Identification No.
Tarring begun
Tarring stopped
Days
100
200
300
Metas. from mamg. tumor
2nd prim. tumor
Metas. from tar tumor
Operated mice with recurrence
Operated mice - no recurrence
7
41
11
44
12
50
16
33
24
23
14
10
3
8
46
15
32
51
47
42
19
9
37
35
40
17
6
39
30
21
2
4
34
48
13
49
5
28
25
45
20
1
27
18
31
22
36
29
Lung
"
Lung + Liver
Ovary
Papilloma from tar
Carcinoma from tar
Recurrence of mammary gland tumor

TEXT-FIG. 3.

whether individuals which already had developed tumors would behave differently in response to tar.

Since mice with spontaneous tumors of the mammary gland rarely live more than 2 or 3 months, most of them could not be expected to survive the period of tar painting (4 months) unless the tumors were removed. It is difficult to obtain a large number of operable tumors at any one time, and so the collection of mice with suitable tumors was begun in the fall and continued several months. 48 of them were at length acquired, from a number of different strains. More than half were agouti; and most of the remainder were albino, though a few were chocolate, cinnamon, silver, or spotted. The tumor ablations were performed under ether by Dr. James B. Murphy and Mr. Ernest Sturm, to whom acknowledgement is here made. The growths were taken out soon after the tumor had been discovered. When the wound was healed and the mice in good condition, painting 3 times a week with coal tar extract was begun and continued for 120

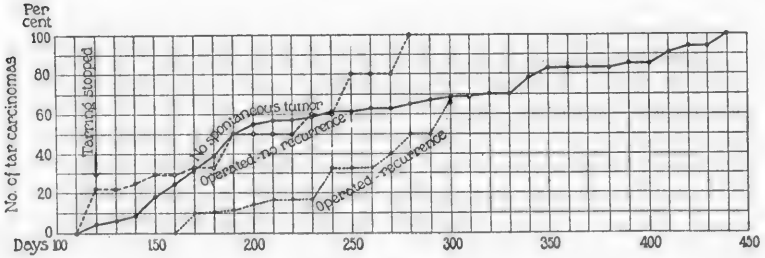


TEXT-FIG. 4.

days as in the experiment already described. With the mice first operated upon, treatment was begun in December, and the others were subjected to it at intervals, the last of them in April. This unavoidable spreading of the experiment over several months necessitated a second group of albinos as controls, tarred synchronously with the mice last operated upon, in addition to the groups with high and low tumor incidence, on which treatment had been started in November.

More of the mice submitted to operation died before the end of the tarring than of those not so treated even when the spontaneous tumor did not recur. By the 120th day only 28 of the mice operated upon were living compared with 49 in the group without spontaneous tumors. This was probably a result of the treatment received. The tumor mice were somewhat older than those without spontaneous tumors but not sufficiently so for age to have been an important factor

in the fatalities. Several of the mice operated upon developed papillomas after tarring earlier than did any in the control groups (50th to 70th day as against the 80th or 90th) (Text-figs. 1 and 3), and papillomas continued to be more frequent up to the 150th day. On the other hand the percentage of carcinoma developing in the operated series lagged behind that of the mice without spontaneous tumors until the 280th day. These differences are apparent from a comparison of Text-figs. 1 and 3. Text-fig. 4 affords a comparison of the percentage of incidence of carcinomas found at successive periods in the two groups of mice. The percentages are calculated as in the preceding graph. Although the series operated upon lagged behind the control the difference is not very marked. It will be seen that in general they behaved in a very similar way. If, however, we



TEXT-FIG. 5.

distinguish in the series operated upon between those mice which did and those which did not have recurrences of the spontaneous mammary gland tumor, and compare the developments with those in the control mice, we find (Text-figs. 1 and 3) that it is the animals with recurrences which develop papillomas earlier, whereas malignant transformation rarely occurred in them. Of the 16 mice with recurrences which lived more than 120 days only 2 ever yielded carcinomas while 5 of the 9 which showed no recurrence eventually developed such growths. Text-fig. 5 shows the percentage of carcinoma at successive periods occurring among the three groups of mice: (1) those which had no recurrence, (2) those from which a mammary gland tumor had been removed and which did have a recurrence, and (3) those which had not developed a mammary gland tumor. The total number of

individuals in each group is very small so that the results are not conclusive but a difference in the response given by the mice with recurrences seems to be indicated. The first carcinomas in the series operated upon which showed no recurrences appeared at the 120th day when 2 of the 9 survivors (22 per cent) showed induration. In the non-operated group 2 carcinomas had appeared at this time (4 per cent) among the 49 surviving animals. None had occurred in the 19 mice operated upon which developed a recurrence then or later. Indeed, in the series last mentioned no carcinoma was found until the 170th day when 1 occurred in the group of 11 individuals as contrasted with 14 out of 44 (32 per cent) and 2 out of 6 (33 per cent) in the other 2 series. Unfortunately the mortality from the tarring was rather high among the mice from which the spontaneous tumors had been removed, so that even fewer individuals are represented in the later figures. However, the persistent absence of tar carcinomas in the mice with recurrences would seem important. At 230 days only 1 had died of such a carcinoma and 5 were still free from it (though 4 had papillomas), giving a group incidence of 17 per cent, whereas among those without recurrence 3 out of 5,—a total which includes both the living animals and those already dead from the neoplasm,—or 60 per cent, had developed a malignant growth. The control mice which had not been operated upon also showed a high incidence of malignancy, the record showing that 24 out of 41 of them (58 per cent) had tar tumors. At the 260th day there continued to be fewer tumors among the mice that had recurrences (2 out of 6) than among the mice without recurrences (4 out of 5) and also among the control animals (26 out of 41). By the 310th day all the mice with recurrences had died. The 2 mice without recurrences which were still living both had developed tar tumors.

The 2 oldest survivors among the animals with recurrences died after 290 and 300 days respectively without having developed more than a papilloma. Among the control animals 4 lived from 400 to 430 days from the beginning of the tarring without developing a neoplasm; but about the 410th day 1 of them developed a carcinoma where the tar had been applied, thus showing that a delay of as much as 290 days may elapse before there is a neoplastic response.

As already stated microscopic examinations were made of the

growths found at autopsy save in a few cases in which postmortem changes or other causes made it impossible. The malignant tar tumors proved to be carcinomas except in one instance (No. 18 in the operated group) which was a carcinosarcoma. Metastasis was found in 7 cases, usually to the lung but in No. 3, an albino, the mediastinum, diaphragm, and thoracic wall were also involved; and in No. 18 a metastasis from the sarcomatous portion of the tar tumor was found in the spleen. A number of tumors developed in regions outside the painted area. The only 2 of them in the mammary gland appeared in the agouti strain which ordinarily has a high incidence of mammary gland tumors. One was found in Mouse 7 which had not had a tar epithelioma, whereas the other developed in Mouse 2 about 100 days after the tar tumor had been first recorded. It is evident that a tumor produced by tar does not suffice to protect the host absolutely against the development of a spontaneous tumor in another organ. Male 13 which had developed in response to the tarring a small papilloma that persisted for only a short time, was very much later seen to have an infiltrating epithelioma of the jaw. Since mice remove tar from a painted area by licking it off it is perhaps surprising that epithelioma on the jaw or tongue is not more frequently encountered. In the case mentioned the neoplasm could not certainly be regarded as a result of the tarring since jaw tumors occur spontaneously in the strain. In two other cases the ovary was the site of a carcinoma but the most frequent region of occurrence for tumors developing at a distance from the tarred region was the lung. It is interesting to note that of the 23 pulmonary growths found none was in the agouti or high tumor strain in which the incidence of spontaneous lung tumors is very low.

SUMMARY AND DISCUSSION.

1. Two strains of mice, one with a high, the other with a comparatively low incidence of spontaneous tumors of the mammary gland, when painted between the shoulders with coal tar extract developed tar tumors with about practically identical frequency.

Possibly this result was to have been expected. If tumors of certain organs or tissues are specific in heredity, a conception for which there is some evidence, then assuredly a high degree of incidence for one tissue, as for example the mammary gland, does not necessarily mean

a high incidence for another tissue, such as the skin, when subjected to tarring. To test the influence of heredity on the response of the skin to tar painting it would be best to employ strains of animals exhibiting differences in the incidence of spontaneous cancer of the skin. Unfortunately no such material was available. We can state on the basis of our experiments only that no hereditary differences in the response of the skin to tar painting were demonstrable in two strains of mice manifesting markedly different percentage incidences for spontaneous mammary growths. It is possible, of course, that the natural differences in the two strains may have been wiped out by the tar treatment. Such a view is suggested by the result of other work in this laboratory which has shown that tar painting increases markedly the incidence of tumors of the lung[6] and destroys the resistance to transplantable tumors (unpublished work).

2. Mice from which spontaneous mammary tumors had been removed were treated with tar. The percentage incidence of the resulting tar tumors was similar to that met with in the controls, except possibly in the case of such animals as showed a recurrence of the spontaneous growth. In them the development of tar tumors seemed to be delayed and possibly prevented. The numbers involved are too small to be conclusive.

Murray[7] has stated that "the induction of a fresh primary growth after a first has been definitely established, meets with a very intense resistance whether the first tumor be of the same parent tissue or another." His conclusions are based partly upon certain retarring experiments but more especially upon a series of mice from which spontaneous mammary gland tumors had been removed, which were then tarred. Apparently these latter did not have recurrences of the spontaneous growth. Only 1 of them developed a papilloma and none carcinoma. The tar was applied twice weekly in Murray's experiment instead of 3 times as in ours and for this as for many other reasons a direct comparison is not possible. It is furthermore not clear what the expectation for his group would have been. Our

[6] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1925, xlii, 693.

[7] Murray, J. A., *8th Scient. Rep. Imperial Cancer Research Fund*, London, 1923, 75.

conclusions, however, seem only partly to confirm his for we find that if any protection exists against a tar cancer after the ablation of a spontaneous mammary growth, it is only when a recurrence has taken place. From certain additional tarring experiments Murray concluded[8] that if a second tarring is begun before cancer has developed from the first, susceptibility is increased. Truffi[9] also has found a diminished refractoriness after tarring. Among our mice in which the mammary gland recurrence appeared during or after the tar painting, no increase in susceptibility but rather the reverse was found. Future experimentation must determine to which among several variables this divergence of results is to be attributed.

[8] Murray, J. A., *Brit. Med. J.*, 1924, ii, 1004.
[9] Truffi, M., *Riforma med.*, 1924, xl, 985.

SEROLOGICAL STUDIES ON THE BLOOD OF THE PRIMATES.*

I. The Differentiation of Human and Anthropoid Bloods.

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(Received for publication, June 18, 1925.)

The problem of man's kinship to his closest relatives in the animal kingdom has been studied by the methods of comparative anatomy and paleontology and the results of these investigations are developing into a special branch of learning. When serology provided a new technique for recognizing the biochemical properties which characterize species it became inevitable that this technique should be applied to the problem of man's ancestry.

The serological procedure which has been chiefly followed is that of the precipitin reaction.

Grünbaum[1] made the first experiments with anthropoid blood. He found that anti-human serum gave a precipitate with the blood of the gorilla, the orang-utang, and the chimpanzee "practically indistinguishable from that obtained with human blood either in quality or quantity. Occasionally it has seemed that the blood of the orang gave a more gelatinous precipitate as compared with the granular precipitate of the other bloods, but this may have been due to accidental circumstances." He prepared immune sera against gorilla, orang, and chimpanzee bloods and was "unable to assert that there is any difference of reaction amongst the many combinations of anti-serum and blood which can be made with the four above-mentioned bloods and sera." In consideration of these conclusions it seems difficult to interpret the apparently specific reactions observed by him microscopically and mentioned incidentally.

The well known studies of Nuttall[2] on the precipitin reaction had for their object the correlation of the serological properties of the serum protein of

* A preliminary communication has appeared in *Science* (Landsteiner, K., and Miller, C. P., Jr., *Science*, 1925, lxi, 492).

[1] Grünbaum, A. S. F., *Lancet*, 1902, i, 143.

[2] Nuttall, G. H. F., Blood immunity and blood relationship, Cambridge, 1904.

animals with their position in the zoological system. Using several anti-human sera and one each of anti-chimpanzee, anti-orang, and anti-baboon serum, Nuttall made qualitative tests on the serum[3] of a great number of animals, including primates.

With anti-human immune serum he obtained positive reactions with 100 per cent of the specimens of man and anthropoid apes examined, 92 per cent of the Old World monkeys (Cercopithecidæ), 78 per cent of the New World monkeys (Cebidæ), and 50 per cent of the marmosets (Hapalidæ). He found that the occurrence of positive reactions was directly proportional to the proximity to man as indicated by the usual criteria of classification.

Nuttall also made a smaller series of tests in which he estimated the volume of precipitate. With chimpanzee serum the volume was 130 per cent of that obtained with human serum, but the precipitate was less compact; with gorilla serum, 64 per cent; with orang serum, 42 per cent; with mandrill serum, 42 per cent; with baboon serum, 29 per cent; and with the serum of one of the New World monkeys, 29 per cent. Nuttall remarks that the figure for the orang is probably too low. Since, however, in precipitin reactions the amount of precipitate has a maximum at a certain concentration of the antigen, the estimation of this amount at an arbitrary dilution does not exactly measure the activity of a given serum. No statement was made by Nuttall as to the possibility of differentiating with certainty human and anthropoid serum by the precipitin reaction.

According to Chiò[4] anti-human serum reacts equally well on human and orang-utang serum. His results with anti-monkey serum were likewise in agreement with Nuttall's. Friedenthal[5] also asserts that the serological differentiation of man and the anthropoid apes cannot be made by any method. He had previously[6] tried to investigate the relationship of species by observing the occurrence or non-occurrence of hemoglobinuria after heterologous transfusions. The existence of individual differences alone makes this method unreliable. He noted that an injection of 25 cc. of human blood into a chimpanzee produced no symptoms. Friedenthal also reported that the blood of lower monkeys was hemolyzed *in vitro* by human serum and human blood by monkey serum.

Marshall[7] made a more careful study of human and monkey blood by means of normal and immune hemolytic sera. He found that normal heterologous sera (goat, sheep, ox, goose, and rabbit) were practically equally hemolytic for human and *Macacus* blood. Human anti-erythrocyte immune sera hemolyzed the bloods of *Macacus rhesus* and *Macacus cynomolgus* almost as well as human bloods, while

[3] In the original publications the term blood is commonly used since in many cases solutions of blood were used for the tests. The tests, however, do not involve the blood cells but the serum protein.

[4] Chiò, M., *Atti r. Accad. sc.*, 1905–06, xli, 1093.

[5] Friedenthal, H., *Arch. Physiol.*, 1905, 1.

[6] Friedenthal, H., *Arch. Physiol.*, 1900, 494.

[7] Marshall, H. T., *J. Exp. Med.*, 1901–05, vi, 347.

anti-monkey serum was only feebly hemolytic for the human bloods examined. Differences between the two kinds of blood were also found by absorption experiments. Nevertheless, Marshall concluded that "there is quite a close relationship between the corpuscles of human beings and both of the varieties of monkeys examined."

There has been a considerable amount of work done[8–13] on the differentiation of human and monkey proteins, especially with regard to the forensic identification of blood stains. The results agree with those of Nuttall in showing that anti-human sera react with monkey serum, but less intensely than with human serum. The specificity of the reactions can be increased by previous saturation with monkey serum.[14,15]

One observation by von Dungern and Hirschfeld[16] may be considered as indicating a difference between human and chimpanzee blood. These authors tested the action of human Group III serum after absorption with human Group II blood, on the red cells of one chimpanzee. The agglutinins of three such sera were completely removed for chimpanzee and for human blood. But one serum thus treated would still agglutinate chimpanzee blood and none of several human bloods. It is impossible to decide with certainty from this observation whether the findings bespeak a constant species difference. (See the following paper of this series, pages 854 and 856.)

We know of no additional work on the serological properties of the blood of anthropoids since the paper of von Dungern and Hirschfeld (1911).

It is possible that the precipitin technique can be improved so as to yield additional information on the interrelationship of the primates. Another method which suggests itself is the use of anti-erythrocyte sera. In spite of the great amount of work on hemolysis and hemagglutination, these reactions have never been systematically exploited in the study of species relationship as has the precipitin reaction. The explanation for this neglect lies apparently in the presumption that the species specificities of the precipitins and of the hemagglutinins are of the same order and that species specificity means protein specific-

[8] Wassermann, A., and Schütze, A., *Berl. klin. Woch.*, 1901, xxxviii, 187.
[9] Uhlenhuth, *Deutsch. med. Woch.*, 1902, xxviii, 659, 679.
[10] Biondi, C., *Vierteljährschrift gerichtl. Med.*, 1902, xxiii, suppl. 1.
[11] Layton, E. N., *Tr. Chicago Path. Soc.*, 1903, v, 217.
[12] Ewing, J., *Proc. New York Path. Soc.*, 1903–04, iii, 14.
[13] Stern, R., *Deutsch. med. Woch.*, 1901, xxvii, 135.
[14] Fujiwara, K., *Deutsch. Z. ges. gerichtl. Med.*, 1922, i, 754.
[15] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1924, xl, 91.
[16] von Dungern and Hirschfeld, *Z. Immunitätsforsch., Orig.*, 1910–11, viii, 526.

ity. It was tacitly assumed that the results in the two types of reaction would run parallel. Recently it has been pointed out (Landsteiner and van der Scheer[15,17,18]) that this supposition is unwarranted. As had been surmised before, the antigens which engender antibodies against red blood cells do not consist simply of proteins. It is possible to extract from erythrocytes by means of alcohol non-protein substances, possibly of a lipoid nature, which have binding and immunizing properties. Certain inconsistencies regarding the antigens of red corpuscles have been reconciled.[17]

Considering the chemical dissimilarities of corresponding antigens, it is not surprising to find differences in the specificity of antibodies against the serum proteins and those against the red blood cells. One of the most striking and for our present purpose most important observations is this, that anti-erythrocyte immune bodies will regularly differentiate between species so closely related that their sera are indistinguishable by means of the precipitin reaction.[15,17] We have therefore undertaken to study the blood cells of the anthropoid apes by means of anti-erythrocyte sera.

EXPERIMENTAL.

Methods.—The two methods which we have mostly employed have been: first, the determination of the hemagglutinin titers of anti-erythrocyte sera on the blood cells of man and monkeys, and second, the determination of the fraction of agglutinin which remained after absorption by red blood cells of the various species. The titrations were made in the usual way in series of tubes containing decreasing concentrations (by halves) of inactivated immune serum. Each tube contained 0.5 cc. of the solution to which was added 1 drop of a 2.5 per cent suspension of washed blood cells. The readings were usually made after 1 or 2 hours at room temperature. For the absorptions, the immune sera, each suitably diluted according to its strength, were mixed with one-half volume of packed, washed red cells and allowed to stand at room temperature (up to 2 hours), during which time they were occasionally mixed by inversion of the tubes, and then kept in the refrigerator overnight. They were then centrifuged and the supernatant fluids titrated as described above.

The immune sera were prepared in the usual manner by a series of intravenous and intraperitoneal injections into rabbits of washed erythrocytes. Anti-human immune sera were prepared against blood cells of Groups I, II, and III.

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[17] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1925, xli, 427; xlii, 123.

[18] Landsteiner, K., and van der Scheer, J., *J. Immunol.*, 1924, ix, 213.

Comparison of Human and Anthropoid Bloods.

Comparison of the titers of several anti-human immune sera for human and chimpanzee bloods respectively yielded no constant differences. Often the titers for chimpanzee blood were appreciably lower than for the human; but differences in the opposite direction

TABLE I.

Titration of Anti-Human Immune Sera with Human and Chimpanzee Erythrocytes.

	Group.	Cells.		
		Human.		Chimpanzee E.
		Group I.	Group II.	
		Highest effective dilution.		
Immune Serum 24	I	200	200	100
" " 26	I	500	250	375
" " 420	II	500	750	750
" " 421	II	3000	2000	2000

Reading after 2 hours at room temperature. Readings of all weak reactions were made microscopically in this as well as in the other experiments.

TABLE II.

Agglutination of Human and Chimpanzee Erythrocytes by Anti-Human Serum after Absorption with Chimpanzee Cells.

Anti-human erythrocyte serum 23 (Group I) (1/20 dilution) absorbed with cells of Chimpanzee F.

Human blood cells.																							Chimpanzee E.	Chimpanzee F.
	33	34	35	36	37	38	39	40	18	25	28	8	W	5	6	7	8	9	10	11	12	13		
Group.	I	II	II	I	II	I	II	II	I	IV	I	III	II	I	I	I	II	IV	II	I	II	II		
	+±*	+	+±	+±	+	+	+	+	+	+	±	+	++	+	+	+±	+	+	+±	+	+	+	0	0

Tests made in small tubes with the following quantities.

1 drop absorbed immune serum.

1 drop salt solution.

1 drop 2.5 per cent suspension of washed blood cells.

Readings made after 2 hours at room temperature.

* In this and the following tables the signs have the following significance: 0 = negative; F. tr. = faint trace; Tr. = trace; ±, +, +, ±, ++, etc.

also occurred. The titers for human blood were also inconstant probably in part due to the difference in the blood group. The results of an experiment are shown in Table I. For other examples, see the absorption experiments below. Titrations with two anti-chimpanzee immune sera gave distinctly higher titers for the homologous bloods (see Table IV) in most cases.

In one experiment with orang blood the results were comparable to those with chimpanzee blood, while gibbon blood gave a definitely lower titer.

TABLE III.

Titration of Anti-Human Erythrocyte Serum after Absorption with Chimpanzee Cells.

Blood cells.	Anti-human erythrocyte serum 23 (Group I) (1/40 dilution).			Anti-human erythrocyte serum 344 (Group II) (1/20 dilution).		
	Control unabsorbed.	Absorbed with Chimpanzee E.	Absorbed with Chimpanzee F.	Control unabsorbed.	Absorbed with Chimpanzee E.	Absorbed with Chimpanzee F.
	Highest effective dilutions.					
Chimpanzee E.....	750	<40	<40	320	<20	20
" F.....	1000	320	<40	320	80	<20
" H.....	750	40	<40	320	<20	<20
" I.....	500	<80	<40	320	<20	<20
Human 72 (Group I).....	4000	2560	640	1600	1280	320
" 77 (" II).....	2000	640	160	800	320	320
" 78 (" III).....	2000	1280	320	800	640	240
" L (" IV).....	2000	640	320	800	320	240

Reading after 1 hour at room temperature.

Absorption Experiments.—Experiments in which anti-human immune sera were absorbed with chimpanzee erythrocytes and *vice versa* yielded unequivocal and constant results. Such human bloods must be selected which will not leave behind any group-specific agglutinins. After removal of the agglutinins for chimpanzee blood, such liquids always contained agglutinins for human bloods of all groups. We have tested a great number (68) of human bloods in this manner since it became evident that considerable individual variation exists. The bloods of four chimpanzees were used. Table II is given as an example.

To determine the proportion of agglutinins in anti-human immune

serum which act on both human and chimpanzee bloods, and those which are specific for human blood alone, the supernatants of the absorbed sera were titrated. In consideration of the existence of individual differences in human bloods, the fluids were tested on a great number of blood specimens, especially selected for their dissimilarities. Table III shows the findings in a typical absorption experiment.

The tests show that in all cases with appropriate sera a considerable portion of the agglutinating capacity of the serum remained after absorption with the heterologous blood. This portion approximated in

TABLE IV.

Titration of Anti-Chimpanzee Immune Serum after Absorption with Human Blood Cells.

Serum 475 (1/10 dilution) absorbed with human erythrocytes (Group I).			Serum 474 (1/20 dilution) absorbed with human erythrocytes (Group II).		
Blood cells.	Control unabsorbed.	Absorbed.	Blood cells.	Control unabsorbed.	Absorbed.
Human (Group I).....	120	<10	Human (Group I).....	200	<40
" (" I).....	240	<10	" (" II).....	200	<20
" (" I).....	80	<10	" (" III).....	400	20
" (" I).....	120	<10	Chimpanzee E.....	400	120
" (" II).....	80	<10	" F.....	600	160
" (" III).....	60	<10			
Chimpanzee E.....	320	160			

Reading after 1 hour at room temperature.

the case of immune serum 344, 20 to 40 per cent after absorption with blood of Chimpanzee F; 40 to 80 per cent after absorption with blood of Chimpanzee E (see Table V). The fraction acting on both bloods was therefore between 20 and 80 per cent. It will be observed in Table III that individual differences were not limited to human bloods, for the blood of one chimpanzee showed a peculiarity. After absorption with the cells of Chimpanzee E as a rule the immune serum still contained an appreciable quantity of agglutinins for Chimpanzee F. This observation held true for this blood in a number of other tests. Absorption with the cells of Chimpanzee F, on the other hand, removed all of the agglutinins for all of the chimpanzee bloods tested, and in all instances left behind agglutinins for human blood. Analo-

gous experiments were made with several different immune sera. It is quite possible that similar results could be obtained with the serum of normal animals, but we have not made such experiments. Results confirming those reported were obtained when anti-chimpanzee immune sera were absorbed with human blood (see Table IV).

The conclusion to be drawn from these experiments is that it is regularly possible to differentiate human and chimpanzee bloods by means of suitable anti-erythrocyte immune sera.

As we had at our disposal only small quantities of orang blood only one absorption experiment was made on anti-human serum with orang blood. The absorbed liquid was insufficient for titrations, and the tests were made with single drops as in the experiments in Table II. The experiment demonstrated that orang blood differs from human blood and indicated that the relationship between orang and chimpanzee bloods is closer than it is between orang and human. Agglutinins remained for the eight human bloods tested and to a larger degree for that of Chimpanzee F, which was shown before to have an individual peculiarity, while all the agglutinins for the other two chimpanzees and four orangs were absorbed.

It has been claimed by Bruck[19] that the sera of various human races can be differentiated by the complement fixation reaction; but this has been denied by other authors.[20,21] We have examined by the methods employed in the other experiments the blood of negroes. Our experiments show that if serological differences do exist between the bloods of white men and American negroes—no longer a pure race—they are much smaller than those between man and the anthropoid apes. So far we have been unable to demonstrate any characteristic difference. It is not impossible, however, that slight differences might be found if individuals of several races preferably of pure blood were carefully studied by this method in all of its modifications.

Comparison of the Bloods of Man and the Anthropoid Apes with Those of the Lower Monkeys.

In another series of experiments we have studied the serological relationship of the erythrocytes of man and chimpanzee to those

[19] Bruck, C., *Berl. klin. Woch.*, 1907, xliv, 793.

[20] Marshall, H. T., and Teague, O., *Philippine J. Sc., Sect. B*, 1908, iii, 357.

[21] Fitzgerald, J. G., *J. Med. Research*, 1909, xxi, 41.

of the lower monkeys. Table V shows that immunization with the bloods of lower monkeys (baboon and macaque) did not engender any considerable quantity of antibodies against the blood of the higher apes, and *vice versa*. It should be noted that the sera employed in the absorption did not have a very high titer and that some previous experiments showed there was a somewhat greater fraction of agglutinins acting on both bloods in question (see references 7 and 18).

TABLE V.

Titration of Various Anti-Erythrocyte Immune Sera after Absorption with Various Primate Blood Cells.

Bloods.	Anti-human erythrocyte serum 344 (Group II) (1/20 dilution).			Anti-chimpanzee erythrocyte serum 474 (1/20 dilution).			Anti-baboon erythrocyte serum 458 (1/20 dilution).			Anti-macaque erythrocyte serum 456 (1/20 dilution).		
	Control unabsorbed.	Absorbed by baboon cells.	Absorbed by macaque cells.	Control unabsorbed.	Absorbed by baboon cells.	Absorbed by macaque cells.	Control unabsorbed.	Absorbed by human Group I cells.	Absorbed by chimpanzee cells.	Control unabsorbed.	Absorbed by human Group II cells.	Absorbed by chimpanzee cells.
Human (Group II).....	400	400	400	160	160	160	80	<20*	20	60	<20	40
Chimpanzee.....	200	200		640	640	480	80	80	<20	40	20	<20
Baboon.....	80	<20	<20	40	20	<20	600	400	400	800	800	800
Macaque.....	80	40	<20	40	<20	<20	400	300	200	800	800	800

Reading after 1 hour at room temperature.
*Test made with Group I blood.

When absorbed with monkey blood the agglutinating powers of both the anti-human and anti-chimpanzee sera were not markedly affected. A similar result was obtained on treating anti-baboon and anti-macaque sera with human and chimpanzee blood respectively. Also in this experiment a distinct difference between human and chimpanzee blood could not be demonstrated.

Comparison of the Bloods of the Lower Monkeys.

With the method employed above a difference was demonstrable between the two genera of Old World monkeys (Catarrhina) investi-

TABLE VI.

Titration of Anti-Baboon Erythrocyte Serum after Absorption with Macaque Blood Cells.

Serum 458 (1/20 dilution) absorbed with erythrocytes of *Macacus rhesus*.

Bloods.	Control unabsorbed.	Absorbed.
Human Group II	80	80
Chimpanzee F	80	80
Baboon	1000	320
Macaque	500	<20

Reading after 1 hour at room temperature.

TABLE VII.

Titration of Anti-Macaque Erythrocyte Serum after Absorption with Baboon Blood Cells.

Serum 456 (1/20 dilution) absorbed with erythrocytes of baboon.

Bloods.	Control unabsorbed.	Absorbed.
Human (Group I)	40	20
Chimpanzee E	40	20
Baboon	1600	<20
Macaque	800	40

Reading after 1 hour at room temperature.

TABLE VIII.

Titration of Anti-Baboon and Anti-Macaque Immune Sera after Absorption with Sapajou Erythrocytes.

Blood cells.	Anti-baboon immune serum 458 (1/20 dilution). Absorbed with blood cells of sapajou (*Cebus hypoleucus*).		Anti-macaque immune serum 456 (1/20 dilution). Absorbed with blood cells of sapajou (*Cebus hypoleucus*).	
	Control unabsorbed.	Absorbed.	Control unabsorbed.	Absorbed.
Baboon	640	640	640	640
Macaque	640	640	320	320
Sapajou	80	<20	80	<20

Reading after 3 hours at room temperature.

gated, more especially by the use of the antiserum against the baboon in comparison with that against the macaque. This may have been due to an accidental circumstance (see Tables VI and VII).

The differences between the two species of Catarrhina and of one New World monkey—ringtail or sapajou monkey (*Cebus hypoleucus*)—were very marked. In this case the titration alone showed a striking specificity.

The absorption of the anti-baboon and anti-macaque sera with the blood of the sapajou did not diminish the titer for the cells of baboon and macaque (Catarrhina).

DISCUSSION.

The experiments described show that a definite and constant serological difference is demonstrable between the bloods of man and the two anthropoids studied—chimpanzee and orang-utang. The experiments also show that this method of agglutination by absorbed immune sera is suitable for the demonstration of serological differences between the bloods of species so closely related that they are indistinguishable by the precipitin reaction as it is usually employed. The difference between the bloods of the lower monkeys on the one hand and of man and the anthropoids on the other is considerably greater than that between the two latter, as is seen from the titers of the immune sera and the results of absorption experiments. The experiments permit of a further consideration. Both anti-human and anti-chimpanzee serum, after absorption with the blood of a lower monkey, do not lose an appreciable amount of their agglutinin content. Also anti-monkey sera behave quite similarly when treated either with chimpanzee or with human blood. It may be concluded, therefore, that anthropoid blood is not much more similar to that of the lower monkeys than is the blood of man to that of lower monkeys. This conclusion is in agreement with the accepted view of zoologists that man has not evolved directly from any of the existing species of primates, as was formerly supposed, but that the Catarrhina, anthropoids, and man have all sprung from a common stock. (See the diagrammatic representation in the third paper of this series, page 871.)

It is of interest to compare the degree of difference which we have

found between the bloods of man and chimpanzee with that already observed between the bloods of other pairs of closely related animals. In the case of the horse and donkey,[18] for instance, the proportion of homologous agglutinins left after absorption with the heterologous blood is the same or greater than in the case of man and chimpanzee, when one makes allowance for individual differences. Measured by the agglutinin reaction bloods of the latter pair would seem to be no farther distant than those of the horse and donkey, between which hybridization is easily possible. It must not be overlooked, however, that horse and donkey are regarded as considerably closer related morphologically than are man and apes.

SUMMARY.

While the precipitin tests do not differentiate, according to the results of previous workers, between the serum proteins of man and chimpanzee, a clear-cut differentiation between the blood cells of man and the anthropoids was obtained by means of hemagglutinins.

According to our tests on bloods of whites and negroes, constant racial serological differences among human bloods, if they exist at all, are certainly smaller than the differences between the bloods of man and the anthropoid apes.

The serological differences between man and the lower monkeys appear to be no greater than those between the anthropoid apes and the lower monkeys. These findings confirm the opinion that the anthropoid apes do not rank in the genealogical tree between lower monkeys and man.

The authors desire to acknowledge their indebtedness to the following persons for their courtesy in assisting them to secure much of the material examined in these studies: Mr. Ellis Joseph, New York City; Dr. W. Reid Blair, of the New York Zoological Society; Mr. C. Emerson Brown, Superintendent of the Zoological Society of Philadelphia; Mr. George P. Vierheller, Superintendent of the Zoological Garden, St. Louis.

SEROLOGICAL STUDIES ON THE BLOOD OF THE PRIMATES.

II. The Blood Groups in Anthropoid Apes.

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(Received for publication, June 30, 1925.)

Knowledge of the blood groups in man[1] has assumed an anthropological significance as result of the work on their racial distribution by L. and H. Hirschfeld.[2] The studies of these workers, which have been confirmed and amplified by numerous others, have established the fact that the percentages of the four blood groups vary in the different human races. In substance it has been shown that among North Europeans Group II is predominant over Group III, while the opposite ratio holds for Asiatics and Africans. Other striking features are for instance a great preponderance of Group I among North American Indians (Coca and Deibert[3]) and a remarkably low percentage of Group I in Ainos.[4]

The intricate questions of the origin and racial distribution of the blood groups are of sufficient importance to anthropology and ethnology to make worth while the accumulation of all information which may give clues to their solution. An obvious question which presents itself is whether substances identical or similar to those which characterize serologically the human groups are present in the bloods of the other primates.

Observations on this point have been made by von Dungern and Hirschfeld.[5] They studied the blood cells of one chimpanzee. According to these results the

[1] Landsteiner, K., *Wien. klin. Woch.*, 1901, xiv, 1132; Oppenheimer's Handbuch der Biochemie, Jena, 1910, ii, pt. 1, 414.

[2] Hirschfeld, L., and Hirschfeld, H., *Lancet*, 1919, ii, 675.

[3] Coca, A. F., and Deibert, O., *J. Immunol.*, 1923, viii, 487.

[4] Ninomiya, Y., *Tôhoku J. Exp. Med.*, 1925, vi, 266. Grove, E. F., personal communication.

[5] von Dungern and Hirschfeld, *Z. Immunitätsforsch., Orig.*, 1911, viii, 541, 547.

blood of this chimpanzee corresponds closely to the human Group II. Absorption of three human Group III sera with human Group II erythrocytes removed all of the agglutinins for this chimpanzee blood. But after similar treatment of a fourth serum a part of the agglutinins for the chimpanzee cells was left. Two chimpanzee sera were also examined. One of them acted on all human bloods of Group III and on some of Group II but on none of Group I. The other serum contained heteroagglutinins for human bloods, besides group agglutinins for human blood of Group III. If one assumes that the cells belonging to these chimpanzee sera possessed group-specific properties, one of them can be classified as Group II and the other as either Group I or II.

The blood cells of macaque monkeys did not react characteristically when treated with different human sera.

EXPERIMENTAL.

Methods.—The presence in human serum of heteroagglutinins for anthropoid bloods precludes the application to them of the simple routine technique for the grouping of human blood and renders the absorption tests with normal human sera less reliable. We have therefore employed the isoagglutinin reaction only in connection with two other methods, both of which yielded wholly reliable results, while they have the advantage of being applicable to small amounts of blood, a consideration of some moment in the case of our material.

The methods by which the effect of the heteroagglutinins was obviated were: first, employment of purified "agglutinin solutions,"[6] and second, the use of group-specific immune sera[7,8]—described below as Methods B and C respectively.

A. For the simple isoagglutination tests 1 drop each of normal human serum, salt solution, and 2.5 per cent erythrocyte suspension were mixed in small tubes.

B. Preparation of the "agglutinin solutions:" 5 cc. of strongly agglutinating human serum, Group II or III, was mixed with 5 drops of packed, washed human blood cells of Group III or II, respectively, and allowed to stand about 2 hours at room temperature, during which time they were occasionally agitated. The cells were then separated by centrifugalization and washed three times with salt solution. The sediment, which still showed strong agglutination, was taken up in about 0.6 cc. of salt solution and placed in a water bath at 56°C., for not longer than 5 minutes, during which time it was shaken. The clumps were usually broken up by this treatment. The tube was then placed in a large centrifuge tube filled with water of 56°C. and centrifuged a short time to throw down most of the sedi-

[6] Landsteiner, K., *Münch. med. Woch.*, 1902, xlix, 1905.

[7] Landsteiner, K., *Wien. klin. Rundschau*, 1902, No. 40.

[8] Hooker, S. B., and Anderson, L. M., *J. Immunol.*, 1921, vi, 419.

ment as quickly as possible. The supernatant fluid was then removed and centrifuged a longer time unheated. Tests with such agglutinin solutions were made in small tubes as described under A above.

C. Preparation of absorbed immune sera: Group-specific antisera were obtained by the immunization of rabbits with human Group II and Group III cells, respectively.[7,8] Only those sera were employed which were found to contain a sufficient quantity of group-specific immune bodies. An appropriate dilution of

TABLE I.

Agglutination of Chimpanzee and Orang Erythrocytes by Normal Human Serum.

Human sera.	Group.	Blood cells.		
		Chimpanzee E.	Chimpanzee F.	Orang S.
28	I	++	+±	
30	I	+(lysis).	+	
88	I	++		
94	I	++		
16	I			++
96	II	±		
97	II	±		
1	II	0	0	
23	II	Tr.	0	
27	II	+±		
14	II			+(lysis).
17	II			+±
91	III	+++		
93	III	++		
8	III	+++	+++	
J	III	+±	+±	+±
L	IV	Tr.	Lysis.	
G	IV	+		
25	IV	+	+	+

Readings after 1 hour at room temperature.

In this and subsequent tables, the following signs are used: 0 = negative; F. tr. = faint trace; Tr. = trace; ±, +, +±, etc.

the sera depending on the strength (we ordinarily used 1/40 dilution) was mixed with 1/2 volume of washed human Group I blood sediment, allowed to stand 1 hour at room temperature and overnight in the refrigerator, and the supernatant fluid was separated by centrifugalization. Tests were made as described under A above.

D. Grouping with Forssman antiserum: According to the observation of Schiff and Adelsberger[9] certain Forssman heterogenetic antisera (prepared by the im-

[9] Schiff, F., and Adelsberger, L., *Z. Immunitätsforsch.*, 1924, xl, 335.

munization of rabbits with suspensions for instance of horse kidney) react on human Group II cells. The test with one such serum, which was highly specific in agglutinating human Group II erythrocytes, proved to be useful as a confirmation. (The results are not presented in a table.)

The action of normal human serum on chimpanzee and orang cells is illustrated by the experiments given in Table I; while in Table II, the action of chimpanzee serum on human cells is recorded.

It will be seen that most of the tests indicate that the bloods of Chimpanzees E and F may well correspond to the human Group II, a finding which was established by other methods as well. But the unreliability of grouping done in this way is brought out by the fact that human sera of Group II which are rich in heteroagglutinins can act as strongly on chimpanzee erythrocytes as a serum belonging to

TABLE II.

Agglutination of Human Erythrocytes by Chimpanzee Serum.

	Human blood cells.								
	M	94	95	96	97	98	H	93	L
Group	I	I	II	II	II	II	II	III	IV
Serum Chimpanzee E	Tr.	±	0	Tr.	F.tr.	±	Tr.	++	+±

Reading after 1 hour at room temperature.

Group III. Grouping of orang blood by the method is still less certain. If it is to be used at all for grouping chimpanzee blood one must select such sera as contain very strongly active isoagglutinins.

The tests have a bearing on the question of the differentiation of human and anthropoid bloods though we observed an instance of agglutination of chimpanzee cells by chimpanzee serum which was not in accord with the typical blood grouping. But if one take into account all of the tests performed, they are sufficient to establish the species specificity of human and chimpanzee blood.

Tables III, *a* and *b* give examples of experiments by which a satisfactory grouping of the anthropoid bloods was carried out. In the tests on chimpanzee erythrocytes the reactions were either distinctly positive or negative. The same was true of tests with orang and gibbon blood and purified normal agglutinins. In the tests on orang blood with absorbed immune sera the effect could be seen from the

TABLE III*a*.

Agglutination of Orang and Gibbon Erythrocytes by "Agglutinin Solutions" Prepared According to Method B.

Agglutinin solution prepared from	Orang.				Gibbon.	Human.	
	T	U	V	W		II	III
Human Group II serum	0	+	+	0	0	0	+
" " III "	+	0	0	+	+	+	0

TABLE III*b*.

Agglutination of Chimpanzee Erythrocytes by "Agglutinin Solutions" Prepared According to Method B and Group-Specific Immune Sera According to Method C.

Agglutinin solution prepared from	Chimpanzee.						Human.	
	E	L	M	N	O	P	II	III
Human Group II serum	0	0	0	0	0	0	0	+
" " III "	+	+	+	±	+	0	+	0
Immune serum 414 absorbed	+++	+++	++±	++	++±	0	+++	0
" " 481 "	0	0	0	0	0	0	0	+±

Readings after 1 hour at room temperature.

TABLE IV.

Absorption of Sheep Hemolysins by Chimpanzee Blood.

Immune serum 422 (anti-human Group II erythrocyte) in 1/100 dilutions absorbed overnight with one-half volume of sediment of each blood. Tests consisted of:

0.5 cc. supernatant fluid.

0.5 cc. fresh guinea pig serum (1/10 dilution).

1 drop 50 per cent suspension of sheep blood.

Readings after 20 minutes in water bath at 37°C.

	Dilutions.				
	100	200	400	800	1600
Control (unabsorbed)	C	C	AC	D	0
Absorbed with cells of Chimpanzee E	0	0	0	0	0
" " " " " F	0	0	0	0	0
" " human cells Group I	C	C	AC	D	0
" " " " " II	0	0	0	0	0
" " " " " III	C	C	AC	D	0

C indicates complete hemolysis; AC, almost complete hemolysis; D, distinct hemolysis; 0, no hemolysis.

difference in the action of the immune serum absorbed with human Group I blood and absorbed with human Group II blood. These tests show that with the proper technique chimpanzee and orang bloods can be grouped as exactly as human blood.

No distinct isoagglutinin reactions were observed in the blood of ten *Macacus rhesus* monkeys and four baboons (*Papio papio*).

The experiments with immune serum against the Forssman antigen showed that the similarity existing between chimpanzee and human blood of Group II extends even to the fact that both these cells contain a factor related to the Forssman antigen.

This was also brought out by the following observation: As Schiff and Adelsberger[9] have shown, certain, not all, rabbit immune sera for human Group II red cells contain hemolysins for sheep cells, which can be removed by absorption with human blood of Group II or IV but not by blood of Group I or III. Table IV shows that this hemolysin is absorbed by chimpanzee blood belonging to Group II, as readily as by human Group II blood.

It is well known that in human blood the isoagglutinins follow the remarkable rule that a blood serum contains isoagglutinins for those isoagglutinogens which are absent from its own cells. This fact has given rise to discussion on the origin and inheritance of the normal antibodies. It has been of interest to see whether a similar rule held for the anthropoids. As we had at our disposal only small quantities of anthropoid serum, the number of experiments which were made to investigate this point is not large. In all cases, however, in which tests could be made, the isoagglutinins were found to follow the same rule as for human blood (see Tables V and VI).

The faint reactions of chimpanzee serum on orang blood of the same group are probably weak heteroagglutinin reactions. It is noteworthy that the blood of Chimpanzee F was weakly, but definitely agglutinated by the serum of Chimpanzee E although both belonged to the same group (Group II). This shows that besides the typical group differences other individual differences exist. We are not in a position to decide whether or not the two animals concerned belonged to different varieties.

The group reactions which we have observed by the various methods in anthropoid bloods correspond in every respect to the group reactions

TABLE V.

Isoagglutination Reactions with Anthropoid Bloods.
Action of Normal Chimpanzee Serum on Chimpanzee Erythrocytes.

Sera.	Chimpanzee erythrocytes.						
	E	F	H	K	L	M	O
	Group.						
	II	II	II	I	II	II	II
Serum Chimpanzee E	0	±	0	0	0	0	0
" " F	0	0	0	0			
" " H	0	0	0	0			
" " K	++	++±	++±	0			

Action of Normal Orang Serum on Orang Erythrocytes.

Sera.	Orang erythrocytes.			
	T	U	V	W
	Group.			
	II	III	III	II
Serum Orang U	+	0	0	++
" " V	+	0	0	+±
" " W	0	+	±	0

Readings after 1 hour at room temperature.

TABLE VI.

Action of Normal Chimpanzee Serum on Orang and Gibbon Erythrocytes.

Sera.	Erythrocytes.					
	Orang.				Gibbon.	Chimpanzee E.
	T	U	V	W		
	Group.					
	II	III	III	II	II	II
Chimpanzee E	Tr.	+±	+±	Tr.	0	0
" F	0	+	+	Tr.	0	0

Readings after 1 hour at room temperature.

in human blood. This is significant in connection with the tests on the bloods of the lower monkeys reported in a communication to follow.[10]

According to one observation of von Dungern and Hirschfeld[5] the chimpanzee blood did not absorb all the isoagglutinins from human serum III. In our experiments, however, which were repeated several times, the human isoagglutinins were practically entirely absorbed by chimpanzee cells.

The typings of the anthropoid bloods according to the foregoing experiments may be summarized as follows: of fourteen chimpanzees three belong to Group I and eleven to Group II as they exist in human blood. Of the two sera and one blood of chimpanzee studied by von Dungern and Hirschfeld,[5] two specimens apparently belong to Group II. The third is considered by these authors to belong also to Group II but it might possibly be assigned to Group I. If we adopt the view of von Dungern and Hirschfeld and add their determinations to ours, a total of seventeen chimpanzees have been grouped, of which three had blood of Group I and fourteen of Group II.

Our results with the orangs are different. Of six individuals two belong to Group II, three to Group III, and one to Group IV. The one gibbon (*Hylobates lar*) examined belonged to Group II.[11]

DISCUSSION.

From the foregoing experiments it may be concluded that the bloods of the anthropoid apes contain groups identical with those of human blood. We were able to assign each blood to one of the four human groups. The occurrence of the agglutinogens of the human type seems to be limited to man and the anthropoids, for the serological factors related to the human isoagglutinogens which are detectable in the blood cells of the lower monkeys and other mammals can be differentiated from the human factors by means of reactions with group-specific immune sera.

The presence of the blood group factors in the apes has evidently a

[10] Landsteiner, K., and Miller, C. P., Jr., *J. Exp. Med.*, 1925, xlii, 863.

[11] This finding was incorrectly reported in our preliminary communication (*Science*, 1925, lxi, 492) as Group III.

bearing on the question of the significance and origin of the blood groups in man. The literature on the subject[2,12–16] contains two chief hypotheses, one that the blood groups were present in the different races of primitive man, the other that they arose as relatively recent mutations. If our findings in the anthropoids are taken into consideration, the simplest assumption seems to be that the isoagglutinable factors existed before man and the anthropoids were differentiated from their common ancestor. If this asssumption is not made, one is forced to the conclusion that identical mutations occurred in the evolutionary lines which developed into the gibbon, orang, chimpanzee, and man at some later time.

Paleontological studies have led to the generally accepted conclusion that the existing species of man is only one of a number of human species, the rest of which have become extinct,[17] as for instance the Neanderthal man. If the group factors had evolved before the human stage had been reached they must have been present in these various fossil human species. Either the groups were distributed among them in a manner comparable with the distribution in man of the present day or else in such wise that each species contained only a part of them.

In contemplating the latter case the question presents itself whether or not the crossing of human types more or less pure as regards the groups may have resulted in the puzzling existence in man of a few sharply defined serological qualities and in their distribution. In this respect the findings in anthropoid apes are of interest. In chimpanzees only the isoagglutinogen A (of Group II cells) was encountered. Although the number of individuals examined is not large, and more extensive studies are needed, it is probable that the factor A is either characteristic for these animals or is predominant, and is present in a higher proportion than in any known race of man. A parallel case,

[12] Hirschfeld, L., and Hirschfeld, H., *L'anthropologie*, 1918–19, xxix, 505.

[13] Schiff, F., and Adelsberger, L., *Centr. Bakt., 1. Abt., Orig.*, 1924, xciii, 172.

[14] Amzel, R., Halber, W., and Hirszfeld, L., *Z. Immunitätsforsch.*, 1925, xlii, 369.

[15] Bernstein, F., *Z. Indukt. Abstammungs.- u. Vererbungsl.*, 1925, xxxvii, 237.

[16] Steffan, *Arch. Rassen- u. Ges.-Biol.*, 1923–24, xv, 137.

[17] See Keith, A., The antiquity of man, London, 2nd edition, 1925.

which can be cited, is that of the North American Indians whose bloods are almost entirely of Groups I and II.

Up to the present no essential difference between group-specific and species-specific agglutinins and agglutinogens has been demonstrated with certainty (see the following paper). One may suppose, therefore, that from the crossing of two species which yield fertile hybrids, an F_2 generation might be obtained in which segregation of the parental characters would result in the appearance of blood groups. For the elucidation of these problems it would be valuable to determine which species of animals exhibit well defined blood groups and whether such species can reasonably be supposed to have had a polyphyletic origin.

SUMMARY.

The bloods of twenty-one anthropoid apes have been examined serologically. It was found possible to assign each to one of the four human blood groups.

The isoagglutinogens of the anthropoids were found to be identical with those of human blood. These very same factors could not be demonstrated in the blood of the lower monkeys.

In the blood of the fourteen chimpanzees examined only the isoagglutinogen A has been found, whereas both A and B were present in the blood of the six orangs.

The significance of these findings for the knowledge of the human blood groups is discussed.

SEROLOGICAL STUDIES ON THE BLOOD OF THE PRIMATES.

III. Distribution of Serological Factors Related to Human Isoagglutinogens in the Blood of Lower Monkeys.

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(Received for publication, June 30, 1925.)

It has been shown that agglutinins exist in the sera of various animals, which react on the isoagglutinogens of human erythrocytes, and conversely that there are substances in animal blood cells resembling the human isoagglutinogens.[1] Von Dungern and Hirschfeld found that treatment of normal human serum with the erythrocytes of certain animals, for example the rabbit and the steer, remove a part of the isoagglutinins. Blood cells of other species fail to do this. The factors in the corpuscles which were responsible for the absorption do not completely resemble those in human blood. This is shown by the fact, for instance, that substances are present in rabbit serum which act upon the human agglutinable factor B,[2] but which, as is to be expected, do not react on rabbit blood.

Related findings on the similarity of the factor A to the Forssman antigen have been made by Schiff and Adelsberger.[3]

[1] Landsteiner, K., *Wien. klin. Rundschau*, 1902, No. 40. von Dungern and Hirschfeld, *Z. Immunitätsforsch., Orig.*, 1910–11, viii, 526. Hooker, S. B., and Anderson, L. M., *J. Immunol.*, 1921, vi, 419. Halpern, J., *Z. Immunitätsforsch., Orig.*, 1911, xi, 609.

[2] We designate the isoagglutinable factors of human erythrocytes by the usual symbols: A for that in Group II cells; B for that in Group III cells.

[3] Schiff, F., and Adelsberger, L., *Centr. Bakt., 1. Abt., Orig.*, 1924, xciii, 172; *Z. Immunitätsforsch.*, 1924, xl, 335. Hesser, S., *Acta med. Scand.*, 1924, suppl. 9. Amzel, R., Halber, W., and Hirszfeld, L., *Z. Immunitätsforsch.*, 1925, xlii, 369.

We have undertaken to search for group factors in the bloods of the lower monkeys for two reasons; first, in order to compare the findings with those obtained with the bloods of the anthropoid apes and already reported;[4] secondly, because we considered it desirable to determine by examination of a number of species of certain families of animals whether the presence of the serological factors in the blood cells of animals bears any general relationship to their position in the systematic zoological classification.

It has been pointed out in preceding papers[4-7] that the relationships thus far established between the zoological classification of animals and their serological qualities differ for proteins on the one hand and the agglutinogens and lysinogens of cells, especially erythrocytes, on the other. The relationship is not as evident in the latter case as in the former, the zoological distribution of the serological factors appearing rather irregular. Yet it does not follow that laws of some kind will not be found.

EXPERIMENTAL.

Methods.—The absorption method was not suitable for our purpose because we often had at disposal only small quantities of blood. This method has the additional disadvantage of measuring only the gross differences between treated and untreated sera, not giving clear-cut results when but a small fraction of the antibody is absorbed. We have, for these reasons, employed the technique used in the experiments on the anthropoid apes; namely, agglutination tests by means of "purified agglutinin solutions," absorbed group-specific immune sera, and a heterogenetic (Forssman) antiserum. These methods are described as Methods B, C, and D in the preceding communication.[4]

In Table I are given some representative tests on monkey bloods. The following list contains the species examined, arranged according to families and genera. The number of individual members of each species tested appears in parentheses whenever that number exceeds unity.

[4] Landsteiner, K., and Miller, C. P., Jr., *J. Exp. Med.*, 1925, xlii, 853.
[5] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1924, xl, 91.
[6] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1925, xlii, 123.
[7] Landsteiner, K., and Miller, C. P., Jr., *J. Exp. Med.*, 1925, xlii, 841.

TABLE I.

*Agglutination Tests on Monkey Erythrocytes.**

The agglutinin solutions were prepared from human Group II and Group III sera according to Method B.

The immune sera were group-specific, anti-human, erythrocyte sera (Group II and Group III), which had been absorbed with human Group I cells according to Method C. Serum 54 was a heterogenetic (Forssman) antiserum prepared by the immunization of rabbits with horse kidney (Method D).

	Macacus rhesus.	*Macacus rhesus.*	Mangabey.	Moustached monkey.	Pig-tailed monkey.	Bonnet monkey.	Green monkey.	Vervet monkey.	Sapajou.	Sapajou.	Woolly monkey.	Howler.	Lemur.	Chimpanzee (Group II).	Human (Group II).	Human (Group III).
Agglutinin solution from human Group II serum	0	F.tr.	0	0	0	0	0	0	++	+±	+	+	+	0	0	+
Agglutinin solution from human Group III serum	0	0	0	0	0	0	0	0	0	Tr.	0	0	F. tr.	+	+	0
Absorbed immune serum for Group II	0	0	0	0	0	0	0	0	0	Tr.	0	0	+	+++	+++	0
" " " " " III	0	0	0	0	0	0	0	0	0	Tr.	0	0	0	0	0	+±
" " " 54 (heterogenetic)	Tr.	F.tr.	0	0	0	0	±	+	0	Tr.	Tr.	+	++	++	++	±

Readings after 2 hours at room temperature.

* In the tables the following signs are used: 0 = negative; F. tr. = faint trace of agglutination; Tr. = trace; ±, +, +±, etc.

List of Monkeys.

	No. examined.
I. Old World Monkeys. Cercopithecidæ.	
Baboons—Genus *Papio.*	
Guinea baboon.....*P. papio* or *P. sphinx.*	(3)
Mantled " .....*P. hamadryas.*	
Species uncertain; either *P. porcarius* or *P. cynocephalus.*	(4)
Guenon Monkeys. Genus *Cercopithecus.*	
Green monkey.....*C. sabæus.*	(2)
Vervet " .....*C. pygerythrus.*	(3)
Patas " .....*C. patas.*	
Mona " .....*C. mona.*	(3)
Moustached " .....*C. cephus.*	
Hochuer " .....*C. nictitans.*	
Mangabeys. Genus *Cercocebus.*	
Sooty mangabey.....*C. fuliginosus.*	(2)
White-collared mangabey.....*C. collaris.*	
Gray-cheeked " .....*C. albigena.*	
Macaque Monkeys. Genus *Macacus.*	
M. rhesus.....	(16)
Java monkey.....*M. cynomolgus.*	(2)
Lion-tailed monkey.....*M. silenus.*	
Bonnet monkey.....*M. sinicus.*	(2)
Pig-tailed monkey.....*M. nemestrinus.*	
Black macaque.....*M. maurus.*	
II. New World Monkeys. Platyrrhina.	
Family Cebidæ.	
Sapajous, or Capuchin Monkeys. Genus *Cebus.*	
Brown sapajou .....*C. fatuellus.*	(2)
Weeper " .....*C. capucinus.*	(5)
White-fronted sapajou.....*C. albifrons.*	
White-throated " .....*C. hypoleucus.*	
Black-footed *Cebus*.....	
Woolly Monkeys. Genus *Lagothrix.*	
Humboldt's woolly monkey.....*L. lagotrica.*	(3)
Spider Monkeys. Genus *Ateles.*	
Black spider monkey.....*A. ater.*	(3)
Black-headed spider monkey.....*A. cucullatus.*	
Howling Monkeys. Genus *Mycetes.*	
Red howler.....*M. seniculus.*	
Squirrel Monkeys. Genus *Chrysothrix.*	
Chrysothrix (species uncertain; probably *sciúrea*).	
Douroucolis. Genus *Nyctipithecus.*	
Three-banded douroucoli.....*N. trivirgatus.*	
Family Hapalidæ.	
Marmosets.	
Common marmoset.....*Hapale jacchus.*	(3)

Lemurs.
Family Lemuridæ, of the suborder Lemuroidea.
True Lemurs. Genus *Lemur*.

Mungoose lemur	*L. mungos.*	(2)
White-fronted lemur	*L. albifrons.*	
Black lemur	*L. macaco.*	(2)
Ring-tailed lemur	*L. catta.*	
Red-brown "	*L. rufifrons.*	
Spotted lemur		

In addition the bloods of two mandrills (*Papio maimon*) and one drill (*Papio leucophæus*) were obtained, but they could not be satisfactorily examined because for unknown reasons spontaneous clumping of the cells occurred in suspensions of the washed cells in saline.

A total of 76 individuals of 36 species has been examined with the following results:

The 46 individuals of the 18 species of Old World monkeys of the family Cercopithecidæ (Catarrhina) gave, in almost all instances, entirely negative reactions with the agglutinin solutions prepared from normal human Group II and Group III serum. The rare faint reactions which occurred may or may not have been due to imperfections in the technique.

In striking contrast to the results with the Cercopithecidæ were those obtained with the New World monkeys (Platyrrhina). All of the 22 individuals of the 12 species examined gave strongly, or at least distinctly, positive reactions[8] with the agglutinin solution Group II. With the absorbed group-specific immune serum they gave either negative or very weak reactions, which latter, in our opinion, are not due to group-specific antibodies, but rather to the heteroagglutinins present in the (rabbit) sera.

In the same manner the bloods of some members of the family of lemurs (Lemuroidea), of which 8 individuals of 6 species were examined, reacted positively with the Group II agglutinin solution.

The blood of 3 or 4 species of lemurs examined reacted positively with Forssman antiserum. The agglutination of 2 of these bloods by Group II immune serum may be connected with this reaction. In one case which was negative with Forss-

[8] Only a weak reaction was obtained with one blood of douroucoli (*Nyctipithecus trivirgatus*), not contained in the list, the only instance in which blood of a dead animal was used.

man antiserum a moderate agglutination took place with Group III immune serum. The significance of this reaction could not be determined by absorption tests because of lack of material.

For comparison absorption experiments were carried out in some cases. The results were in complete agreement with those obtained with our routine method. An example is given in Table II, which shows that the blood of the two Platyrrhina (sapajous) absorbed almost the whole agglutinin content of the normal human Group II

TABLE II.

Absorption of Normal Human Serum by Monkey Bloods.

First absorption: Normal human serum (Group II) absorbed for 1 hour at room temperature and overnight in the ice box with one-half volume of packed, washed sediment of each monkey blood. Tests made as follows:

0.05 cc. absorbed serum.
0.1 cc. salt solution.
0.1 cc. 2.5 per cent suspension of human Group III erythrocytes.

Second absorption: Supernatant absorbed again for 1 hour at room temperature with one-fourth volume of packed sediment of each monkey blood. Tests made as follows:

0.1 cc. absorbed serum.
0.1 cc. salt solution.
0.1 cc. 2.5 per cent suspension of human Group III erythrocytes.

Human serum Group II.	Erythrocytes used for absorption.				
	Macaque.	Baboon.	Sapajou.	Sapajou.	
Before absorption					+±
After 1st absorption	+±	+±	F.tr.	F.tr.	
" 2nd "	+±	+±	Tr.	F.tr.	

serum, while the blood of the two Cercopithecidæ (baboon and macaque) failed to diminish the agglutinin reaction.

Titration of the agglutinin solution for Group II against one blood each of the Cercopithecidæ and Platyrrhina (macaque and sapajou) showed that in the case of the former no reaction occurred with 6 drops of the solution, while the smallest quantity which still agglutinated the latter was 1 drop of a 1/8 dilution.

In addition to the tests on monkeys the bloods of other animals have been examined in the same manner as the monkey bloods. Some examples of these tests are given in Table III.

TABLE III.

Agglutination Tests on Erythrocytes of Other Animals.

For methods see the legend to Table I.

	Mouse.	Rabbit.	Rabbit.	Rat.	Guinea pig.	Duck.	Pigeon.	Goose.	Donkey.	Horse.	Pony.	Goat.	Cat.	Dog.	Human II.	Human III.
Agglutinin solution from human Group II serum	+	+±	+±	+±	+	0	0	0	+	0	0	0	+	+±	0	+
Agglutinin solution from human Group III serum	0	Tr.?	+	Tr.	0	0	0	0	0	0	0	0	0	Tr.?	+	0
Absorbed immune serum for Group II	0	0	0	Tr.	0	+	0	±	0	0	0	0	0	Tr.	+++	0
" " " " " III	0	0	0	0	0	+	0	Tr.	0	0	0	0	0	0	0	+±

Readings after 2 hours at room temperature.

Tests with the agglutinin solution prepared from human Group II serum gave distinctly positive reactions on the bloods of rabbit, guinea pig, rat, mouse, cat, dog, steer, donkey, and pig, and negative reactions on sheep, horse, goat, pigeon, chicken, duck, and goose bloods. The Group III agglutinin solution gave positive reactions only on the bloods of rabbit and sheep, and negative reactions on those of all the rest. The occurrence in this series of a considerable number of positive reactions with the Group II agglutinin solution is in accordance with the findings of von Dungern and Hirschfeld already mentioned. These authors found distinct individual differences by means of absorption experiments, but in a limited number of such tests we found either no difference, or, at the most, only slight ones.

DISCUSSION.

It follows from the results described that, as has been previously shown, the isoagglutinins of normal human serum act upon the red blood cells of a considerable number of animals, and the conclusion may therefore be drawn that serological substances similar to the human isoagglutinogens have a widespread distribution in the animal kingdom. But it has also been demonstrated by means of immune sera[9] from rabbits that the structures in the human and animal erythrocytes, though similar, are not identical. For the absorbed group-specific immune sera failed to give any definite reactions with the animal erythrocytes susceptible to the action of normal human isoagglutinins. In our studies only the blood of the anthropoid apes gave such reactions, a fact attesting to the close relationship with man.[10]

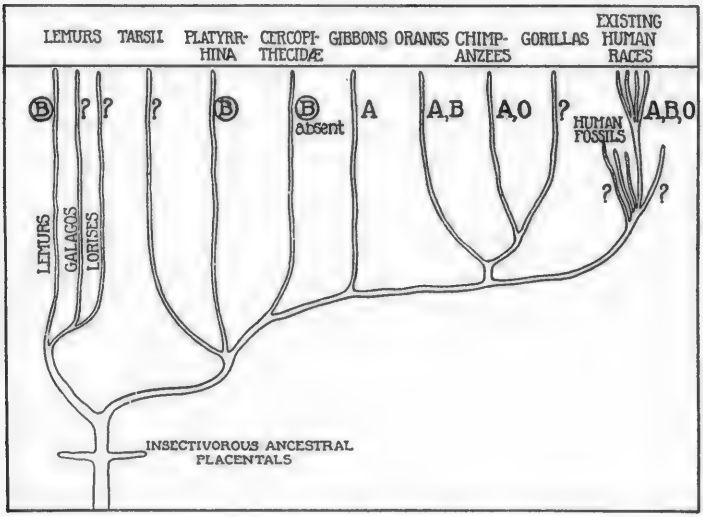
The bloods of ducks and geese reacted with both Group II and Group III immune sera, the former moderately, the latter slightly. But these reactions of duck blood were found to be due to heteroagglutinins which did not correspond to the group agglutinins, for the reactions persisted after treatment of the sera with human Group II and Group III cells. No test of the sort was made with goose blood.

The investigation of the bloods of the lower monkeys brought out an unexpected regularity in the distribution of agglutinogens. Up to the present the zoological distribution of the few agglutinable factors

[9] Sera obtained by the immunization of chickens behaved in a different way. The problem presented by this difference will not be considered here.

[10] It is possible though not probable that the one case mentioned above of a lemur constitutes an exception.

studied by previous workers has, on the whole, appeared to be quite irregular. To be sure, the factor related to B had been found only in mammals, not in birds, but a very small number of species of the latter



TEXT-FIG. 1. Adapted from Keith, A., The antiquity of man, London, 2nd edition, 1925, and Sonntag, Ch. F., The morphology and evolution of the apes and man, London, 1924.

A = agglutinogen of human Group II red cells.
B = agglutinogen of human Group III red cells.
Encircled B = agglutinogen similar to, but not identical with, B.
O = blood and serum corresponding to human Group I.
? = not examined.
Of the gibbons only one individual was examined.

have been examined. Also the Forssman antigen is usually regarded as exemplifying the irregularity of the distribution of receptors.[11]

[11] Possibly a law is indicated by the fact that some very closely related species are similar in that the Forssman antigen is present in their blood (Schmidt, H., Die heterogenetischen Hammelblutantikörper und ihre Antigene, Leipsic, 1924). In this connection it may be mentioned that a positive agglutination was obtained with the blood of three species of lemurs tested with Forssman antiserum prepared by the injection of horse kidney.

In view of these facts it is noteworthy that we have found by our studies in monkeys that whole genera or families appear to be characterized by the presence of certain serological factors (so called receptors) in their erythrocytes. A factor similar to B was found in all of the bloods of the family Platyrrhina examined (genera *Cebus*, *Lagothrix*, *Ateles*, *Mycetes*, *Chrysothrix*, *Nyctipithecus*), in the family of the marmosets (Hapalidæ), and also in the family of lemurs (Lemuridæ). This factor was not found in the blood of the Cercopithecidæ, of which the following genera were examined (*Papio*, *Cercopithecus*, *Cercocebus*, and *Macacus*). While it is obviously impossible to maintain that exceptions may not be found subsequently the uniformity of our results thus far would seem indicative of a definite rule. A peculiarity of the phenomenon unlike the phenomena of specificity in precipitin reactions on serum proteins is the lack of a gradual transition from one family to another—its sharp discontinuity. This point is evident from Text-fig. 1 in which the findings on the group factors in primates are presented.

The results of our studies should stimulate further investigation in the same general direction since it is possible that like zoological relations exist in the case of other serological factors. The human isoagglutinin lends itself readily to the search for certain agglutinogens. It is possible that with methods resembling ours the zoological distribution of other serological factors can be investigated.

SUMMARY.

Serological studies on the bloods of thirty-six species of lower monkeys have shown that there exists a correspondence between the distribution of a certain hemagglutinogen and the place of the species in the zoological system.

In twelve species of seven genera of Platyrrhina (New World monkeys) and six species of the genus *Lemur* a factor similar to the human isoagglutinogen B was present; in eighteen species of four genera of Cercopithecidæ (Old World monkeys) it was absent, although the latter are more closely related to man than the former.

It would seem from our findings that a genus, perhaps even a family, of animals may be characterized by a special serological factor. The factor found in the lower monkeys is not identical with the one existing in the erythrocytes of the anthropoid apes and man.

STUDIES OF LEPTOSPIRA HEBDOMADIS.

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(Received for publication, June 16, 1925.)

Besides infectious jaundice (Weil's disease), there prevails in Japan an endemic disease which is symptomatically closely related to infectious jaundice but epidemiologically distinct: seven day fever (the *nanukayami*, of Fukuoka province), or autumn fever (*akiyami*, of Shizuoka province). Inada concluded from an epidemiological study of the two diseases that they were etiologically distinct. The isolation of *Leptospira icterohæmorrhagiæ* from infectious jaundice was accomplished by Inada and Ido in 1914, and in 1917 Ido, Ito, and Wani isolated a somewhat similar organism, *Leptospira hebdomadis*, from *nanukayami*. The biological properties of *Leptospira hebdomadis* were not determined, however, and in 1922 an expedition was sent to Shizuoka province for the study of *akiyami*. Numerous cases, in some years more than 100, occur in the region of the Oi River. The endemic season is practically limited to the period between August and October, the seasonal prevalence of *akiyami* being similar to that of *nanukayami* and quite different from that of infectious jaundice. The average morbidity is about 0.3 per cent. The infection occurs chiefly in persons engaged in cultivation of the soil. Most of the patients are under 20 years of age.

Thirty cases of *akiyami* were studied, clinically and bacteriologically. The date of the first case was September 6, of the last, October 13. Two types of *Leptospira hebdomadis* were isolated during this study. The characteristics of the two types will be discussed further on.

Isolation of the Leptospira.

In the isolation of the leptospira from *akiyami* patients we followed the original method of Inada and injected blood from the patients intraperitoneally into young guinea pigs (150 to 200 gm.). Some of the animals showed the typical

TABLE I.

Transmission from Patients to Guinea Pigs.

Case No.	Date of onset.	Day of disease on which blood was taken.	Direct culture from blood.	Amount of blood injected.	No. of guinea pigs inoculated.	Leptospira in blood of guinea pig.	Course after infection.	Autopsy. Jaundice.	Autopsy. Hemorrhage.	Autopsy. Leptospira in liver.	Results of subsequent passage.
	1922			*cc.*							
1	Sept. 6	3rd		2.0	2	+	D. in 8 days. " " 7 "	++ ++	+++ +++	+++ +++	Always +
2	" 12	2nd		2.0	2	−	Remained well.				
3	" 19	3rd		2.0	2	−	" "				
4	" 20	2nd		2.0	2	−	" "				
5	" 26	4th		2.0	1	+	Killed in 10 days.				− 2nd generation.
6	" 27	3rd		1.5	2	+ −	" " 7 " Remained well.	−	++	+	− 3rd "
7	" 28	4th		1.0	2	+ −	Killed in 10 days. Remained well.	−	++	+	− 2nd "
8	" 29	4th		1.5	2	− −	" " " "				
9	" 30	4th		1.5	2	+ +	D. in 9 days. " " 9 "	+++ ++	+++ +++	+++ +++	Always +

10	Sept. 30	4th		1.5	2	+ ?	D. in 8 days. Remained well.	−	+	+	
11	" 30	3rd		1.5	2	+ +	Killed in 7 days. Remained well.	−	++	+	− 2nd generation.
12	Oct. 1	3rd		1.5	2	+ +	" " D. in 9 days.	− −	++ ++	+ +	
13	" 3	2nd		1.5	2	+ +	" " 9 " " " 12 "	− −	+ +	− −	 − 2nd "
14	" 6	3rd		1.5	1	?	" " 6 "	−	++	+	− 5th "
15	" 10	3rd	+	2.0	1	+	" " 6 "	−	+	+	− 3rd "
16	" 11	1st	+	1.5	2	+	Remained well. Lost.				
17	" 12	3rd		2.0	2	+	D. Lost.	−	++	+	− 2nd "
18	" 13	4th	+	1.5	2	+ +	D. in 9 days. " " 9 "	++ ++	+++ +++	++ ++	} Always +
19	" 13	3rd	+	1.5	2	 +	Lost. D. in 8 days.	 −	 ++	 +	 − 2nd generation.

In the tables D. indicates died; S., survived.

akiyami symptoms within 1 week. Daily examinations of the blood for the presence of the leptospira were made by means of the dark-field microscope. When the finding was positive, a sample of blood for cultivation was taken directly by heart puncture, without killing the animal. In the case of dead animals, liver emulsion was used for transmission. In some instances a few drops of the patient's blood were mixed with Ringer's solution-agar medium, and incubated at room temperature, or 30°C. Examinations were made after 7 to 10 days incubation.

Of nineteen cases which were examined by one or the other of these methods within 4 days after the beginning of the disease, the leptospira was found in fifteen. The details of the transmission experiments are recorded in Table I.

Several facts noted during the animal experiments deserve special attention. In some instances the animals show intense jaundice and hemorrhage, and the infection terminates fatally after a brief illness. In the liver emulsion of such animals a large number of the leptospiras are found. In some of the fatal instances only a slight jaundice may be observed; hemorrhage in the lungs is sometimes marked; the leptospiras in the liver are few in number, and the virulence of the strain diminishes gradually in two or three generations. In this respect the infection resembles very much that of experimental seven day fever (*nanukayami*). In other cases the animals infected with patients' blood show the presence of the leptospira in the blood but survive. These facts had already been noted by Kitamura, who, however, did not determine whether the mildness of the infection is due to variations in the virulence of the strains or to individual differences in the susceptibility of guinea pigs. From the results of our own repeated inoculation experiments, we regard such variations in the experimental disease as due to differences in virulence in the strains of spirochetes.

We obtained pure cultures of the spirochete in twelve of the nineteen cases; three of these (Nos. 1, 9, and 18) were of rather high virulence. The virulence of a given strain is not necessarily parallel to the clinical symptoms of the patient.

We have tentatively classified the *akiyami* leptospiras into two types, according to their virulence in animals, the more highly virulent A type, and the less virulent B type. Comparative immunological

studies have been carried out with *Leptospira icterohæmorrhagiæ* on the one hand and with *Leptospira hebdomadis* on the other.

Immunological Studies.

Pfeiffer's Phenomenon with the Sera of Convalescents.

Blood was withdrawn from sixteen convalescents, within 2 to 4 weeks after defervescence, and 1 cc. of the serum was mixed with 1 cc. of liver emulsion which

TABLE II.

Summary of Pfeiffer Reactions.

Strain of leptospira.	*Akiyami* convalescent sera.															Infectious jaundice.		Anti-*icterohæmorrhagiæ* serum (horse).
	S.M.	H.I.	S.K.	I.T.	M.T.	T.S.	G.Y.	R.T.	T.O.	T.T.	G.W.	J.O.	S.I.	G.K.	K.S.	I.A.			
	Day of disease on which serum was taken.																Case 1.	Case 2.	
	37	22	21	27	21	21	28	33		28	20	31							
Akiyami leptospira A type.																			
Strain 1 (Case 1).....	+	+	+	−		−			−		−	−	−				−	−	−
" 2 (" 9).....	+	+	+			−	−	−					−				−	−	−
" 3 (" 18).....	+	+	+	−	−	−			−	−							−	−	−
Akiyami leptospira B type.																			
Strain 1 (Case 6).....	−	+?	−		+	+			+		+	+	+	+	+	+	−		
" 2 (" 16).....			−						+	+		+		+					
" 3 (" 15).....	−						+								+				
Leptospira icterohæmorrhagiæ (Strain1).....	−	−	−			−	−										+	+	+
Leptospira hebdomadis (Strain 1).....	−				+							+		−					

showed by dark-field examination about 10 leptospiras per field. The mixtures were injected intraperitoneally into guinea pigs, and after 30 minutes, and again after 2 hours, liquid was withdrawn from the peritoneal cavity and examined microscopically for the presence of the organisms. The animals were observed for some time after this examination, and in the event of death autopsies were carried out and the pathological changes studied.

In the case of the B type, owing to the difficulty of obtaining suitable liver emulsions containing these organisms, we used Ringer's solution cultures of the respective strains in their most suitable stages of development. 1 cc. of culture was used in each Pfeiffer test. The examination of the peritoneal liquid was made 30 minutes, 2, 4, and 6 hours after injection.

The results of Pfeiffer tests are summarized in Table II.

A positive Pfeiffer reaction against the A type organism was obtained only with the sera of the patients from whom we had isolated the A type. Convalescent sera from other *akiyami* cases and from infectious jaundice had no effect whatever upon the A type organism. Conversely, we obtained positive reactions upon *Leptospira icterohæmorrhagiæ* with convalescent sera from infectious jaundice cases, but no effect at all with sera from *akiyami* cases.

The *akiyami* convalescent sera which reacted positively with the B type *akiyami* organism gave positive reactions also with *Leptospira hebdomadis*. The convalescent sera which reacted positively against the A type *akiyami* spirochete gave negative Pfeiffer reactions with the B type organism and with *Leptospira hebdomadis*.

As the table shows, the B type of *akiyami* leptospira seems to be identical with *Leptospira hebdomadis*, while the A type is serologically distinct from *Leptospira hebdomadis* as well as from *Leptospira icterohæmorrhagiæ*.

Preparation of Specific Immune Sera.

Monovalent immune sera were prepared in rabbits against each of the *akiyami* strains. We also used in the experiments the serum of a horse immunized against *Leptospira icterohæmorrhagiæ*, prepared in the Government Institute for Infectious Diseases of Tokyo Imperial University.

The immunization of rabbits was usually carried out according to Ōba's method. Sometimes Uhlenhuth's technique was used in the case of the A type leptospira and *Leptospira icterohæmorrhagiæ*. Occasionally intravenous injections were made of the leptospira cultures, but with rather less satisfactory results.

The technique of Ōba's method, namely the injection of culture intrahepatically, can be performed without laparotomy by using a rather long syringe needle. The culture should be injected very slowly. The rabbits injected gradually became emaciated; the development of jaundice depends on the virulence of injected spirochete antigen. The specific agglutinins appear in the serum within 4 to 6 weeks after injection.

When Uhlenhuth's method was used, we injected a large quantity

of liver emulsion intraperitoneally and intravenously at the same time. When the inoculation is successful, the animals show typical symptoms, and jaundice becomes very evident.

Agglutination Reactions.

The technique of agglutination reaction is far more difficult with spirochetes than with most bacteria. Ōba in 1921 published a simplification of his original technique of agglutinin determination *in vitro* for application to *Leptospira icterohæmorrhagiæ*. The details are as follows:

TABLE III.

Agglutination Reactions with Immune Serum against A Type Leptospira (Strain 3).

Test-tube No.	Mixture of: 0.4 per cent agar.	+ anti-*hebdomadis* A immune serum.		Final dilution of serum.	Tested against: *Akiyami* leptospira A type (Strain 1).	*Akiyami* leptospira A type (Strain 3).	*Leptospira icterohæmorrhagiæ* (Strain 1).
	cc.		cc.				
1	2.0	Undiluted.	0.5	1:5	+	+	−
2	2.0	1:2 dilution in normal rabbit serum.	0.5	1:10	+	+	−
3	2.0	1:4 " " " " "	0.5	1:20	+	+	−
4	2.0	1:8 " " " " "	0.5	1:40	+	+	−
5	2.0	1:16 " " " " "	0.5	1:80	+	+	−
6	2.0	1:32 " " " " "	0.5	1:160	+	+	−
7	2.0	1:64 " " " " "	0.5	1:320	+	+	−
8	2.0	1:128 " " " " "	0.5	1:640	+	+	−
9	2.0	1:256 " " " " "	0.5	1:1280	−	−	−
10	2.0	Normal rabbit serum.	0.5	Control.	−	−	−

+, agglutination and degeneration; −, normal growth, no effect.

2 cc. of sterile 0.3 per cent Ringer's solution-agar is distributed into a series of test-tubes, which are then resterilized. 1 cc. of rabbit immune serum is taken up into a syringe with a long needle, 0.5 cc. of which is put into the first tube; 0.5 cc. of normal rabbit serum is taken up into the syringe to dilute the immune serum 1:2; 0.5 cc. of twofold diluted immune serum is delivered into the second test-tube; this procedure is repeated throughout the series of tubes. The serum is

mixed with the melted agar. Before coagulation 1 or 2 drops of spirochete-containing material is added to each tube. The tubes are placed at 37°C. After 3 or 4 days incubation, examinations are made by means of dark-ground illumination.

In the case of positive reaction the multiplied organisms lose their motility and are agglutinated into masses. In the strongly positive instances almost no individual spirochetes can be recognized in the conglomerate masses, which have a

TABLE IV.

Agglutination with A Type Immune Sera.

Strain of leptospira.	Serum 1 (titer 1:320).						Serum 3 (titer 1:640).						
	Dilution of serum.												
	1:10	1:40	1:80	1:160	1:320	Control.	1:10	1:20	1:80	1:160	1:320	1:640	Control.
A type leptospira.													
Strain 1 (Case 1)	+	+	+	+	+	−	+	+	+	+	+	+	−
" 2 (" 9)							+	+	+	+	+	+	−
" 3 (" 18)	+	+	+	+	+	−	+	+	+	+	+	+	−
B type leptospira.													
Strain 1 (Case 6)	−		−		−	−	−	−	−	−	−	−	−
" 2 (" 16)	−	−	−	−		−							
" 3 (" 15)	−	−	−			−							
" 4 (" 5)		−		−		−							
Leptospira icterohæmorrhagiæ.													
Strain 1	−	−	−	−	−	−	−	−	−	−	−	−	−
" 2	−	−	−	−	−	−							
" 3		−		−	−	−							
Leptospira hebdomadis.													
Strain 1	−	−	−	−	−	−							
" 2							−	−	−	−	−	−	−

granular appearance in the center. In a weakly positive reaction, the multiplied organisms show slight motility, especially in the peripheral part of the agglomerates. In all cases microscopic examination is necessary to avoid any possible errors in the examination.

The results of the agglutination tests of all the leptospira strains against monovalent sera prepared with the A type (two strains), the

B type (three strains), *Leptospira icterohæmorrhagiæ* (one strain), and *Leptospira hebdomadis* (one strain) are summarized in Tables III to VI.

It may be safely concluded from the results shown in these tables that the leptospira can be serologically identified by the method used. All four strains of *Leptospira icterohæmorrhagiæ* behave serologically

TABLE V.

Agglutination with B Type Immune Sera.

Strain of leptospira.	Serum 1 (titer 1:160).					Serum 3 (titer 1:320).					
	Dilution of serum.										
	1:10	1:40	1:80	1:160	Control.	1:10	1:20	1:40	1:80	1:160	1:320	Control.
A type leptospira.												
Strain 1	−	−	−	−	−							
" 3	−	−	−	−	−							
B type leptospira.												
Strain 1	+	+	+	+	−	+	+	+	+	+	+	−
" 2						+	+	+	+			−
" 3	+	+	+	+	−	+	+	+	+	+	+	−
" 4						+	+	+	+			−
" 5	+	+	+	+	−	+	+	+	+			−
Leptospira icterohæmorrhagiæ.												
Strain 1	−	−	−	−	−							
" 2	−	−	−	−	−							
" 3	−	−	−		−							
Leptospira hebdomadis.												
Strain 1	+	+	+	+	−	+	+	+	+	+	+	−

exactly in the same manner; they can be readily differentiated from the three strains of *akiyami* A type organism on the one hand, and from the *akiyami* B type and *Leptospira hebdomadis* on the other.

Protective Property of the Immune Sera.

The immune rabbit sera used in this part of the work were prepared by Uhlenhuth's method. An immune horse serum against *Leptospira icterohæmorrhagiæ,*

without preservative, was also utilized. The sera were diluted 1:10, 1:20, 1:50, and 1:100, and 1.0 cc. of a given immune serum or its dilution was mixed with 1.0 cc. of liver emulsion of infected guinea pigs, and the whole amount of the mixture was injected into each guinea pig. In case of death, liver emulsion was examined by means of the dark-field microscope.

TABLE VI.

Agglutination with Immune Sera against Leptospira icterohæmorrhagiæ and Leptospira hebdomadis.

Strain of leptospira.	Anti-*icterohæmorrhagiæ* immune serum (titer 1:320).							Anti-*hebdomadis* immune serum (titer 1:160).					
	Dilution of serum.												
	1:10	1:20	1:40	1:80	1:160	1:320	Control.	1:10	1:20	1:40	1:80	1:160	Control.
A type leptospira.													
Strain 1	−	−					−	−	−	−	−	−	−
" 3	−	−	−				−	−			−		−
B type leptospira.													
Strain 1		−		−	−	−	−	+	+	+	+	+	−
" 2	−	−	−	−	−	−	−						
" 3	−	−	−	−	−	−	−	+	+	+	+	+	−
" 5	−	−	−	−			−						
Leptospira icterohæmorrhagiæ.													
Strain 1	+	+	+	+	+	+	−						
" 2	+	+	+	+		+	−	−	−	−	−	−	−
" 3	+	+	+			+	−						
" 4	+		+	+	+		−						
Leptospira hebdomadis.													
Strain 1	−	−	−	−	−	−	−	+	+	+	+	+	−

The results of one series of the present experiment are shown in Table VII.

The anti-*icterohæmorrhagiæ* immune sera protected guinea pigs against infection with *Leptospira icterohæmorrhagiæ* when given in amounts as small as 0.001 cc., but not against infection with the *akiyami* A type leptospira, even in 1 cc. The *akiyami* A type immune serum protects guinea pigs against the A type infection in 0.01 cc. but not against infection with *Leptospira icterohæmorrhagiæ* in 0.1 cc.

TABLE VII.

Protective Property of Immune Sera.

Anti-*icterohæmorrhagiæ* immune rabbit serum.															Rabbit immune serum against *akiyami* leptospira A type (Strain 3).											
+ *akiyami* leptospira A type (Strain 1).							+ *Leptospira icterohæmorrhagiæ* (Strain 1).								+ *akiyami* leptospira A type (Strain 1).							+ *Leptospira icterohæmorrhagiæ* (Strain 1).					
				Autopsy.							Autopsy.							Autopsy.						Autopsy.				
Amount of immune serum.*	Infective liver emulsion.	Body weight of guinea pig.	Course.	Icterus.	Hemorrhage.	Leptospira in liver.	Amount of immune serum.*	Infective liver emulsion.	Body weight of guinea pig.	Course.	Icterus.	Hemorrhage.	Leptospira in liver.		Amount of immune serum.*	Infective liver emulsion.	Body weight of guinea pig.	Course.	Icterus.	Hemorrhage.	Leptospira in liver.	Amount of immune serum.*	Infective liver emulsion.	Body weight of guinea pig.	Course.	Icterus.	Hemorrhage.	Leptospira in liver.
cc.	*cc.*	*gm.*					*cc.*	*cc.*	*gm.*						*cc.*	*cc.*	*gm.*					*cc.*	*cc.*	*gm.*				
1.0	1.0	170	D. in 7 days.	+	++	+									1.0	1.0	175	S.				1.0	1.0	172	D. in 10 days.	–	–	–
0.1	1.0	162	D. in 6 days.	++	++	++	0.1	1.0	190	S.					0.1	1.0	180	"				0.1	1.0	195	D. in 7 days.	+++	+++	++
0.05	1.0	200	D. in 6 days.	+++	+++	++	0.05	1.0	200	"					0.05	1.0	185	"				0.05	1.0	195	D. in 7 days.	++	++	+
0.02	1.0	180	D. in 6 days.	+++	+++	++	0.02	1.0	204	"					0.02	1.0	180	"				0.02	1.0	195	D. in 3 days.			
0.01	1.0	185	D. in 5 days.	++	+++	++	0.01	1.0	204	"					0.01	1.0	185	"				0.01	1.0	207	D. in 7 days.	+++	+++	++
0	1.0	215	D. in 6 days.	+++	+++	++	0	1.0	207	D. in 7 days.	+++	++	+++		0	1.0	207	D. in 6 days.				0	1.0	205	D. in 6 days.	+++	+++	++

* Ringer's solution was used to bring the amounts of immune serum to a uniform volume of 1 cc.

TABLE VIII.

Protective Property of Immune Sera.

Anti-*icterohæmorrhagiæ* immune horse serum.															Rabbit immune serum against *akiyami* leptospira A type (Strain 3).													
+ *akiyami* leptospira A type (Strain 1).							+ *Leptospira icterohæmorrhagiæ* (Strain 1).								+ *akiyami* leptospira A type (Strain 1).							+ *Leptospira icterohæmorrhagiæ* (Strain 1).						
Amount of immune serum.	Infective liver emulsion.	Body weight of guinea pig.	Course.	Autopsy.			Amount of immune serum.	Infective liver emulsion.	Body weight of guinea pig.	Course.	Autopsy.			Amount of immune serum.	Infective liver emulsion.	Body weight of guinea pig.	Course.	Autopsy.			Amount of immune serum.	Infective liver emulsion.	Body weight of guinea pig.	Course.	Autopsy.		
				Icterus.	Hemorrhage.	Leptospira in liver.					Icterus.	Hemorrhage.	Leptospira in liver.					Icterus.	Hemorrhage.	Leptospira in liver.					Icterus.	Hemorrhage.	Leptospira in liver.
cc.	*cc.*	*gm.*					*cc.*	*cc.*	*gm.*					*cc.*	*cc.*	*gm.*					*cc.*	*cc.*	*gm.*				
1.0	1.0	180	D. in 5 days.	+++	−	+								0.02	1.0	150	S.				1.0	1.0	145	D. in 8 days.	++	+	+
1.0	1.0	200	D. in 2 days.	−	−	−	0.02	1.0	155	S.				0.02	1.0	155	"				1.0	1.0	160	D. in 11 days.	−	−	−
0.5	1.0	200	D. in 5 days.	+++	−	+	0.01	1.0	205	"				0.01	1.0	145	"				0.5	1.0	202	S.			
0.5	1.0	160	D. in 5 days.	+++	+	++	0.01	1.0	155	D. in 19 days.	−	−	−	0.01	1.0	155	D. in 8 days.	−	−	−	0.5	1.0	180	"			
0.2	1.0	204	D. in 7 days.	+++	+	++	0.005	1.0	160	D. in 16 days.	−	−	−	0.005	1.0	160	D. in 11 days.	−	+	+	0.2	1.0	220	D. in 8 days.	+++	+++	++

0.2	1.0	208	D. in 5 days.	+++	+	++	0.005	1.0	140	D. in 16 days.	–	–	–	0.005	1.0	215	S.				0.2	1.0	170	D. in 7 days.	++	+++	++
0.1	1.0	207	D. in 7 days.	+++	++	++	0.002	1.0	215	D. in 11 days.	++	+++	++	0.002	1.0	175	D. in 8 days.	–	+	+	0.1	1.0	190	D. in 8 days.	++	++	++
0.1	1.0	170	D. in 6 days.	+++	++	++								0.002	1.0	152	D. in 11 days.	+++	+++	++	0.1	1.0	152	D. in 8 days.	+++	+++	++
0	1.0	160	D. in 6 days.	+++	+++	+++	0	1.0	210	D. in 6 days.	++	++	++	0	1.0	270	D. in 6 days.	++	+++	++	0	1.0	200	D. in 7 days.	++	+++	++

The guinea pig given 1 cc. of this serum died on the 10th day. The autopsy findings were negative and the cause of death unknown.

In similar experiments (Table VIII) in which relatively smaller amounts of homologous and relatively larger amounts of heterologous sera were used, we obtained nearly the same results as before; that is, 0.005 cc. (1.0 cc. of 1:200 dilution) of anti-*icterohæmorrhagiæ* immune serum protected against the homologous infection, but even 1 cc. did not protect against the A type infection. The A type immune serum always protected in 0.01 cc., and often in 0.005 cc., but the protection was imperfect against heterologous infection even with 1 cc.; (with 0.2 cc. of the immune serum no sign of any positive influence is noticeable).

This experiment brought out a very interesting point with regard to the relation of *Leptospira icterohæmorrhagiæ* and the A type. Though amounts of 1 or 0.5 cc. of anti-*icterohæmorrhagiæ* immune serum do not protect against the A type infection, we did not see at autopsy any marked hemorrhage in the various organs and could find only a few leptospiras in the liver emulsion, while the degree of jaundice was very intense. These findings may possibly indicate a beneficial effect of the immune bodies of the serum, which diminish the action of the infecting organisms, though unable to protect against infection.

The same experiments were carried out six times in succession and always with nearly the same results. We conceive that there is some slight mutual protective influence of the immune sera of *icterohæmorrhagiæ* and the A type when large amounts are used, while remarkable differences in the serological behavior of the organisms are evident when the sera are used in higher dilutions.

Active Immunization.

It has been found that guinea pigs which are susceptible to *Leptospira icterohæmorrhagiæ* can be immunized with dead cultures of the organism. Hence it was of special interest to inoculate guinea pigs with the other types of leptospira in the same way and to test their immunity with homologous as well as heterologous strains. The antigens were prepared as follows:

TABLE IX.

Infection Tests of Guinea Pigs after Active Immunization.

	Antigen.																					
	Leptospira icterohæmorrhagiæ.							*Akiyami* leptospira A type.					*Akiyama* leptospira B type.				*Leptospira hebdomadis.*				Control.	
	Guinea pig No.																					
	1	2	3	4	5	6	7	1	2	3	4	5	1	2	3	4	1	2	3	4	1	2
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.		
Preparatory injections.																						
Apr. 11.....	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5		
" 18.....	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5		
" 25.....	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0		
Subsequent test with infective liver emulsion. May 10	*Leptospira icterohæmorrhagiæ.*			*Akiyami* leptospira A type.				*Leptospira icterohæmorrhagiæ.*			*Akiyami* lepto-spira A type.		*Leptospira ictero-hæmorrhagiæ.*		*Akiyami* leptospira A type.		*Leptospira ictero-hæmorrhagiæ.*		*Akiyami* leptospira A type.		*Leptospira icterohæ-morrhagiæ.*	*Akiyami* leptospira A type.
	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.
Course after injection.....	S.	S.	S.	D. in 6 days.	D. in 5 days.	D. in 4 days.	D. in 6 days.	D. in 6 days.	D. in 5 days.	D. in 7 days.	S.	S.	D. in 5 days.	D. in 5 days.	D. in 6 days.	D. in 5 days.	D. in 5 days.	D. in 7 days.	D. in 6 days.	D. in 6 days.	D. in 6 days.	D. in 5 days.
Autopsy.....				+	+	+	+	+	+	+			+	+	+	+	+	+	+	+	+	+

TABLE X.

Infection Tests of Guinea Pigs after Active Immunization.

	Antigen.															
	Leptospira icterohæmorrhagiæ.							*Akiyami* leptospira A type.					Control.			
	Guinea pig No.															
	1	2	3	4	5	6	7	1	2	3	4	5	1	2	3	4
Preparatory injections.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*				
June 14	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5				
" 20	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0				
" 26	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0				
Subsequent injection with living culture. July 7	*Leptospira icterohæmorrhagiæ.*				*Akiyami* leptospira A type.			*Leptospira icterohæmorrhagiæ.*		*Akiyami* leptospira A type.			*Leptospira icterohæmorrhagiæ.*	*Akiyama* leptospira A type.		
	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.		
Course after injection	S.	S.	S.	S.	D. in 7 days.	D. in 11 days.	S.	D. in 8 days.	S.	S.	S.	S.	D. in 8 days.	D. in 7 days.		
Autopsy					+	+		+					+	+		

Final test with infective liver emulsion. July 22	*Leptospira icterohæmorrhagiæ.*		*Akiyami* leptospira A type.								*Leptospira icterohæmorrhagiæ.*		*Akiyami* leptospira A type.			*Leptospira icterohæmorrhagiæ.*	*Akiyami* leptospira A type.
	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.							1.0 cc.	1.0 cc.	1.0 cc.			1.0 cc.	1.0 cc.
Course after injection.....	S.	S.	D. in 9 days.	D. in 16 days.							D. in 6 days.	S.	S.			D. in 7 days.	D. in 6 days.
Autopsy.....			+	+							+					+	+

Each strain was cultivated in two media, 0.4 per cent Ringer's solution-agar with normal rabbit serum, and Ringer's solution media with normal rabbit serum. At the maximum stage of growth the cultures grown on the agar medium were mixed with the other cultures in proportions of 2:1 and heated for 30 minutes at 57°C. The guinea pigs were given three intraperitoneal injections at 6 to 7 day intervals. 2 weeks after the last injection the degree of active immunity was tested by infection.

Table IX is a summary of the results of immunity tests in one series of such experiments.

It is evident that serological differences exist between *Leptospira icterohæmorrhagiæ* and the *akiyami* A type leptospira.

In another series of guinea pigs we injected living culture following the three injections of vaccine and tested the immunity by subsequent injection of liver emulsion (Table X).

We lost no guinea pigs by using a homologous living strain for the fourth dose, but with one exception all the animals died which received a heterologous living strain on the fourth injection. In guinea pigs actively immunized with a dose of liver emulsion the result was not so precise as before. Nevertheless, we can conclude that rather sharp serological differences exist between *Leptospira icterohæmorrhagiæ* and the A type of leptospira.

Another series of experiments was designed to determine whether or not actively immunized guinea pigs would be able to withstand infection with a heterologous strain.

The preparatory injections were made subcutaneously at 4 day intervals; 1.5 cc. of the vaccine was given at the first injection, 2 cc. at each of three succeeding injections; there followed an injection of 1.5 cc. of living old culture, one of 2.0 cc. of a fresh culture, and, for the seventh and last injection, 1.0 cc. intraperitoneally of liver emulsion of infected guinea pig. The subsequent infection test was performed on the 20th day after the last injection, 1.5 cc. of liver emulsion of a guinea pig infected with heterologous strain being given intraperitoneally. Among the six guinea pigs thus tested, two showed no sign of infection, three developed typical symptoms and died, and the other died of intercurrent infection.

Infection tests of this kind with the B type leptospira and *Leptospira hebdomadis* are impracticable owing to the lower virulence of these organisms and the growth of the guinea pigs to insusceptible adults during the course of the immunization. In these instances Pfeiffer's phenomenon was used as a test of immunity. 3 to 4 cc.

of culture in Ringer's solution of each organism was injected intraperitoneally into actively immunized guinea pigs, and samples of the peritoneal fluid were withdrawn several times at intervals of 30 minutes, 2, 4, and 6 hours, and examined microscopically for the fate of the injected leptospira (Table XI).

The B type of *akiyami* organism and *Leptospira hebdomadis* underwent lysis *in vivo* in the guinea pigs immunized with reciprocal heterologous strains, while neither showed any sign of lysis in guinea pigs immunized against A type or *Leptospira icterohæmorrhagiæ*.

TABLE XI.

Fate of B Type Leptospira and of Leptospira hebdomadis in Peritoneal Cavity of Actively Immunized Guinea Pigs.

Immunized against	Culture injected.	Amount.	Result.
		cc.	
B type leptospira.	B type leptospira (Strain 1).	3	+
A " "	" " " (" 1).	3	−
Leptospira hebdomadis.	" " " (" 1).	3	+
" *icterohæmorrhagiæ.*	" " " (" 1).	3	−
B type leptospira.	*Leptospira hebdomadis.*	4	+
A " "	" "	4	−
Leptospira hebdomadis.	" "	4	+
" *icterohæmorrhagiæ.*	" "	4	−?*
Control (not immunized).	B type leptospira.	3	−
" " "	*Leptospira hebdomadis.*	4	−

* Occasional non-motile leptospiras on first three examinations; none after 6 hours.

DISCUSSION.

As already stated, Inada and Ido had demonstrated in 1914 that *Leptospira icterohæmorrhagiæ* is the causal agent of infectious jaundice in Japan. Uhlenhuth, in 1916, showed independently that Weil's disease of Germany is caused by the same organism, and other observers (Stokes and Ryle, Martin and Pettit, Monti, and others) demonstrated it in cases of infectious jaundice occurring in Europe during the war. Noguchi (1918) isolated two strains of *Leptospira icterohæmorrhagiæ* from wild rats in America and made a thorough study of the biological relations existing between the Japanese, European, and American strains, which proved to be identical. Kaneko, in 1921, also reported that the European and Japanese strains are serologically identical. In 1918, Ido, Ito, and Wani found a

similar organism to be the etiological agent of *nanukayami*, or seven day fever, a disease which might be considered symptomatically as atypical infectious jaundice, but which Inada, in 1910, had concluded was etiologically distinct. This disease is variously known in different parts of Japan as *akiyami* (Shizuoka province), *akinetsu* (Kochi province), and *sakushyunetsu* (Okayama province). The causative organism (*Leptospira hebdomadis*) can be differentiated from *Leptospira icterohæmorrhagiæ* by serological reactions[1] as shown by Ido, and confirmed by Kaneko, and by Ōba, and their etiological identity is generally accepted.

Kitamura, in 1918, reported that the spiral organism which he found in cases of *akiyami* disease is very difficult to differentiate from *Leptospira hebdomadis* and *Leptospira icterohæmorrhagiæ*. Some of the strains which he isolated showed a virulence approaching that of *Leptospira icterohæmorrhagiæ*, others resembled *Leptospira hebdomadis* in this respect. He did not attempt serological identification.

Leptospira icteroides, first discovered by Noguchi, in 1919, in yellow fever cases in Guayaquil, is morphologically practically identical but serologically distinct from *Leptospira icterohæmorrhagiæ*. The behavior of *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ* in Pfeiffer's phenomenon and in agglutination and complement fixation reactions is distinctly different; in active immunization tests the differentiation is less marked. *Leptospira icteroides* is therefore biologically closely related to *Leptospira icterohæmorrhagiæ*, but serologically distinct.

We have found, as originally stated by Kitamura, that there are two types of leptospira causing *akiyami*, types differing in their virulence for guinea pigs. By means of the Pfeiffer reaction with convalescent sera, we have shown that the two strains are serologically distinct and have called the more virulent the A type, and the other the B type. Further serological study showed the B type to be identical with *Leptospira hebdomadis*, of *nanukayami*, while the A type is distinct from both *Leptospira hebdomadis* and *Leptospira icterohæmorrhagiæ*. The serological distinctions among these organisms were marked in Pfeiffer tests and agglutination reactions, but rather slight in active immunization tests. We regard the difference between our A type leptospira and *Leptospira icterohæmorrhagiæ* as probably of about the same degree as that between *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ*.

[1] Noguchi has recently shown that *Leptospira hebdomadis* can be morphologically differentiated from *Leptospira icterohæmorrhagiæ*, when grown under identical conditions, by its greater length and by its straighter body, the ends being hooked only rarely in living specimens.

The Relation of the Akiyami Leptospira in Rats.

That wild rats are the carriers in nature of *Leptospira icterohæmorrhagiæ* and field mice of *Leptospira hebdomadis* has been shown by Inada and Ido. We obtained from the endemic district in which our work was done five wild rats (*Mus decumanus*) and ten field mice (*Microtus montebelli*). The examination of the rats was repeatedly negative, both in urine and kidney tissue, but in the urine of one field

TABLE XII.

Agglutination Reactions with Immune Rabbit Serum Prepared with Leptospira from the Field Mouse.

Strain of leptospira.	Dilution of serum.						Control.
	1:5	1:10	1:20	1:80	1:160	1:320	
Leptospira from the field mouse	+	+	+	+	+	+	−
Akiyami leptospira A type.							
Strain 1	+	+	+	+	+	+	−
" 3	+	+	+	+			−
Akiyami leptospira B type.							
Strain 2	−	−	−	−	−	−	−
" 3	−	−	−	−			−
Leptospira icterohæmorrhagiæ.							
Strain 2	−	−	−	−			−
" 4	−	−	−	−			−
Leptospira hebdomadis.							
Strain 1	−	−	−	−			−

mouse a leptospira was found. The urine was injected intraperitoneally into guinea pigs, which died on the 8th day after injection. Many leptospiras were found in an emulsion of the liver which was inoculated into other guinea pigs. The strain was carried to further generations by inoculation of kidney suspension of the original mouse, and the inoculated animals died on the 17th day after injection. The virulence of this strain is so high that even after artificial cultivation for eight generations it continues to kill guinea pigs of over 450 gm.,

and by immunization of rabbits with the strain we obtained an immune serum of 1:320 titer. This serum was tested against *Leptospira icterohæmorrhagiæ*, *Leptospira hebdomadis* A, and *Leptospira hebdomadis* B, with the results shown in Table XII.

The agglutination reactions, and subsequent protection tests, showed that the field mouse strain is identical with the A type of *Leptospira hebdomadis*. Although only one mouse of the ten was found to harbor the strain, its transmission by field mice (*Microtus montebelli*) is indicated, and further search for the leptospira in field mice in endemic districts is desirable in connection with the epidemiological study of *akiyami*.

SUMMARY.

From the results of etiological study of the disease known as *akiyami* which prevails in the Shida district of Shizuoka province, we conclude that:

1. *Akiyami* is an infectious disease caused by a leptospira.

2. The leptospira causing *akiyami* is very difficult to differentiate morphologically from *Leptospira icterohæmorrhagiæ* and *Leptospira hebdomadis*.

3. The strains of leptospira isolated from cases of *akiyami* are of two types. One type, isolated from three of the sixteen cases, is highly virulent for guinea pigs and is serologically distinct from *Leptospira icterohæmorrhagiæ* and *Leptospira hebdomadis*; we have called this the A type. The type obtained from the other cases is less virulent for guinea pigs and is serologically identical with *Leptospira hebdomadis*; we have called this the B type.

4. The field mouse (*Microtus montebelli*), which harbors *Leptospira hebdomadis*, has also been found to harbor the A type of *akiyami*.

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THE INFLUENCE OF HIGH PROTEIN DIET ON THE KIDNEYS.

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(Received for publication, August 1, 1925.)

The literature contains conflicting reports concerning the effect of excessive protein intake on the kidneys of laboratory animals and man.

Newburgh (1) fed rabbits egg white. It was fed alone to one group, alternated with common stock diet in another group, and mixed with other food in a third group. The time of feeding varied from a few days to 7 months. Casts and albumin appeared in the urine and by serological tests the albumin in the urine was shown to be different from egg albumin. Examination of the kidneys revealed casts in the tubules and varying degrees of tubule damage.

He also fed rabbits 15 to 30 gm. of casein daily, mixed with other articles of diet. Kidney damage was found in rabbits fed 3 weeks, 10 weeks, and 4 months, but one ate the diet for 11 months at which time it had albuminuria but no kidney injury was found by microscopic examination.

Another group of rabbits was fed soy beans and water. The controls were killed at the end of 7 months and had no gross or microscopic evidence of kidney damage. Of the ones fed soy beans, five were killed at the end of 2 months. Four of these were negative, but one had degeneration of tubular epithelium and casts in the lumina. The remaining fifteen were fed 4½ to 12 months longer and eight died. All of these had acute and chronic changes in the tubules, and in five of these no other cause of death than nephritis was found. The remaining seven were killed and all had kidney damage, but it was slight in one animal. The primary effect was on the tubular epithelium and was accompanied by an overgrowth of connective tissue. There was gain in weight. Blood urea determinations on the controls averaged 31 mg. per 100 cc., while those fed soy beans had blood urea of 100 mg. or more per 100 cc. of blood.

Newburgh and Clarkson (2) also found kidney damage in rabbits fed meat. One group of animals ate a diet containing 35 per cent protein and in 6 weeks all had albumin and casts in the urine. They lived from 4 to 7 months. The kidneys were large, the tubules dilated and degenerated. A second group ate a similar diet

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containing 25 per cent protein. Early lesions in these animals were swelling and desquamation of the tubular epithelium. Those living from 24 to 59 weeks had dilatation of the tubules with atrophy and degeneration of the epithelium. The glomeruli were normal and there was no connective tissue increase. Albumin and casts were found in the urine. Nineteen were used as controls, one of which had dilated tubules and scars in the kidney were frequently found.

Similar results are reported by Polvogt, McCollum, and Simmonds (3) who avoided some criticism applicable to Newburgh's work by using rats, because they are omnivorous, and composing a diet containing enough fat, carbohydrate, and accessory food substances for normal growth and reproduction. They varied the protein content by shifting the proportions of the ingredients. The diet consisted of wheat, maize, casein, liver, beans, peas, sodium chloride, calcium carbonate, and butter fat or cod liver oil. The protein content was 31 per cent, 40.9 per cent, 40.6 per cent, and 41.3 per cent in four different arrangements of the above articles. The control diet contained 18 per cent protein. The time of feeding lasted from 129 to 489 days and included five generations. The average blood urea for the controls was 17.6 mg. and that for the others 23.5 mg. per 100 cc. of blood. The kidneys of all except the control group were large, congested, had casts in the tubules, hyaline material in the capsular spaces, and degeneration of the tubular epithelium. There was gain in weight, good growth, and activity of all animals.

Osborne, Mendel, Park, and Darrow (4) also fed rats a high protein diet, but report different results. The articles of diet are not enumerated but the protein content is given as 75 per cent. Weight of these animals increased from 60 to 260 gm. The only kidney change found at the end of the experiment was hypertrophy.

Putnam and Gamble (5) while working on another problem fed rats casein, starch, butter fat, and salt, a diet consisting of 75 per cent protein, and at the end of 2, 3, and 5 months found no retrogressive kidney lesions but there was hypertrophy.

Howe (6) studied the diet of monkeys and found that they do not tolerate over 8 per cent protein. To a group of twenty he fed diets of 18 to 25 per cent protein with enough fat, carbohydrate, and vitamine for their needs. Postmortem examination of these animals included microscopic study and no nephritic changes were found.

Squier and Newburgh (7) studied the effect of high protein diet on five patients with symptoms and signs of beginning chronic nephritis consisting of headache, dizziness, disturbances of vision, high blood pressure, and low phenolsulfonephthalein output. One case, only, had albuminuria. Blood urea nitrogen ranged from 9.8 to 19.1 mg. per 100 cc. of blood. The protein intake was raised from 33 gm. to from 100 to 175 gm. daily, after which red blood cells appeared in the urine of four patients, albumin appeared in three and increased in the one that already had it. Blood urea nitrogen rose to from 15.4 to 28.9 mg. per 100 cc. in three instances, but in one it fell from 15.8 before to 14 mg. while the high protein diet was being taken. Blood pressure was not influenced.

They also studied the effect of feeding two meals of 1½ pounds each of beef steak in a day to each of four normal young men. Red blood cells were found in the urine of every individual and these were taken as evidence of renal irritation.

Procedure.

With these reports as a basis for work the following procedure was planned and carried out.

Young, adult, albino rats were divided into six groups of six each and fed diets containing fat, carbohydrate, protein, salt, and accessory food substances. The ingredients were changed in proportion for each group so as to vary the amount of protein. Obviously, the fat and carbohydrate will also vary in proportion if natural food is used. In one group soy beans furnished practically all of the protein. The food analysis was calculated from tables given in Laboratory handbook for dietetics by Rose. The diet sheets are given below.

	Group No.					
	I	II	III	IV	V	VI
	per cent in gm.	*per cent in gm.*	*per cent in gm.*	*per cent in gm.*	*per cent in gm.*	*per cent in gm.*
Wheat	2.0	30.0	27.0	27.0	—	20.0
Casein	—	1.0	5.0	15.0	—	35.0
Maize	—	—	30.0	30.0	—	—
Orange juice.*	17.0 (cc.)	—	—	—	—	—
Corn starch	70.0	46.0	—	—	19.5	27.0
Dried peas	2.0	10.0	20.0	10.0	—	5.0
Soy beans	1.0	5.0	10.0	10.0	75.0	5.0
Sodium chloride	1.0	1.0	1.0	1.0	1.0	1.0
Calcium carbonate	1.5	1.5	1.5	1.5	1.5	1.5
Butter	5.5	5.5	5.5	5.5	3.0	5.5
Analysis:						
Protein	1.36	9.56	20.17	29.89	28.76	40.13
Carbohydrate	67.80	69.78	55.65	49.90	37.50	42.70
Fat	4.89	6.06	7.42	7.32	13.60	5.82

* Fed separately.

At the beginning of the experiment the left kidney was removed from one rat in each of the above groups save Group I. In this way the load of the remaining kidney was doubled. The food was given *ad libitum*. After 6 months of feeding blood urea nitrogen determinations were made, all remaining animals killed, and complete postmortems performed. Group III was the control.

A seventh group consisted of sixteen pied rats which were fed cooked lean beef

and whole milk. The stock room diet was given to three additional ones for controls. Blood uric acid determinations were made at the beginning and end of the feeding time which was 9 weeks. Then the animals were killed and examined.

RESULTS.

The results can be readily written and much more easily read if put in tabular form as below.

Rat No.	Weight.	Blood urea nitrogen.	Weight of kidneys.			Diameter of tuft.	Diameter of tubule.*	Anteroposterior diameter of kidney.
			Right.	Left.	Body.			
Group I.								
	gm.	*mg. per 100 cc.*	*gm.*	*gm.*	*per cent*	*μ*	*μ*	*mm.*
36†	95.0	—	0.65	0.57	1.28	—	—	—
37	96.0	0.42	0.51	0.47	1.02	78.0	34.2	6.0
38†	87.0	—	0.56	0.57	1.29	—	—	—
39†	71.0	—	0.49	0.52	1.42	—	—	—
40†	72.0	—	0.48	0.45	1.29	—	—	—
47†	87.0	—	0.52	0.50	1.17	—	—	—
Average.....	84.6	0.42	0.53	0.51	1.24	78.0	34.2	6.0
Group II.								
61†	144	—	0.65	0.66	0.90	—	—	—
62†	150	—	0.66	0.62	0.83	—	—	—
65	154	4.83	0.80	0.79	1.03	73.1	35.4	6.0
68†	135	—	0.64	0.61	0.92	—	—	—
69	164	0.42	0.70	0.77	0.89	82.5	33.0	6.0
Average.....	149	2.62	0.69	0.67	0.91	77.8	34.2	6.0
34‡	151	0.84	0.79	—	0.52	94.2	43.5	7.2
Group III.								
70	154	2.80	0.78	0.80	1.02	102.6	40.5	7.5
71	148	4.41	0.70	0.68	0.93	80.1	36.9	7.0
72	164	4.41	0.62	0.60	0.74	95.1	35.7	6.5
73	118	—	0.54	0.50	0.88	103.8	34.2	6.0
74†	115	—	0.53	0.50	0.90	—	—	—
Average.....	140	3.87	0.63	0.61	0.89	95.4	36.8	6.7
75‡	209	1.51	1.13	—	0.54	104.4	42.3	8.0

* Each entry is an average of ten measurements with an ocular micrometer.

† Animal died before end of feeding time.

‡ Animal on which nephrectomy was performed.

Rat No.	Weight.	Blood urea nitrogen.	Weight of kidneys.			Diameter of tuft.	Diameter of tubule.*	Anteroposterior diameter of kidney.
			Right.	Left.	Body.			
Group IV.								
	gm.	*mg. per 100 cc.*	*gm.*	*gm.*	*per cent*	*μ*	*μ*	*mm.*
76	200	3.22	1.20	1.15	1.17	97.5	38.7	7.5
77	261	4.41	1.15	1.10	0.86	107.4	43.2	8.0
78	178	4.06	0.85	0.85	0.95	89.2	36.9	7.0
79	229	3.22	1.10	1.06	0.94	98.1	40.8	8.0
80	165	3.64	0.78	0.75	0.92	89.7	38.4	7.0
Average.....	206	3.71	1.01	0.98	0.97	96.4	39.6	7.5
81‡	204	3.22	1.50	—	0.73	106.2	43.2	9.0
Group V.								
53	210	9.08	0.80	0.85	0.78	90.9	36.6	7.0
54	174	3.64	0.64	0.65	0.74	109.5	34.2	6.5
55	162	4.06	0.79	0.75	0.95	86.1	35.7	7.5
57	142	5.25	0.66	0.61	0.89	89.7	31.8	6.5
59	166	4.76	0.80	0.78	0.95	91.5	33.9	6.8
Average.....	171	5.36	0.74	0.73	0.86	93.5	34.4	6.8
60‡	142	6.86	0.91	—	0.64	103.5	36.0	7.2
Group VI.								
82	140	4.83	0.80	0.85	1.17	107.4	33.9	7.0
83	178	4.83	0.95	0.90	1.03	97.2	39.6	7.5
84	184	8.47	0.84	0.85	0.91	105.2	44.4	7.5
85†	119	—	0.52	0.51	0.86	—	—	—
86	144	5.25	0.84	0.78	1.12	97.5	37.8	7.5
Average.....	153	5.84	0.79	0.78	1.02	101.8	38.9	7.4
87‡	188	8.47	1.10	—	0.58	105.9	40.8	8.0

Complete postmortem examinations were made in every instance and no gross evidence of nephritis was found in any. Microscopic study was made of all kidneys of the animals that lived to the end of the feeding time but no evidence of kidney damage was found in any instance.

Group VII.

Rat No.	Before feeding.		After feeding.	
	Weight.	Blood uric acid.	Weight.	Blood uric acid.
	gm.	*mg. per 100 cc.*	*gm.*	*mg. per 100 cc.*
1	300	3.5	315	3.4
2	250	2.9	250	4.2
3	290	3.0	300	6.8
4	250	3.2	195	3.8
5	300	3.8	310	—
6	185	3.8	245	4.7
7	285	4.1	295	3.0
8	225	3.3	240	3.5
9	230	3.4	225	4.3
10	275	3.4	300	6.0
11	310	3.9	305	3.4
12	260	4.0	Died.	—
13	190	3.8	225	3.7
14	200	4.0	205	2.9
15	210	3.3	245	3.3
16	215	3.0	235	3.2
Average.....	248	3.5	259	4.0
Controls.				
17	190	2.4	210	4.4
18	175	1.7	215	4.7
19	180	2.6	200	2.9
Average.....	181	2.2	208	4.0

No evidence of kidney damage was found by gross and microscopic study.

DISCUSSION.

Omnivorous animals were used and the source of protein was both animal and vegetable.

The animals in Group I were all markedly emaciated. The weight of the kidneys in this group is smallest but the per cent of kidney weight to body weight is largest, indicating that the estimation of kidney hypertrophy by calculating the per cent of kidney weight to body weight is in error because there is no standard of the state of body nourishment.

The average weight of the kidneys in the control group is less than

the figure given by Donaldson (8) but the animals eating from 28.76 to 40.13 per cent protein had kidneys larger than his figure, indicating that there was definite hypertrophy. Additional evidence of hypertrophy is found in the actual measurements of the capillary tufts, convoluted tubules, and anteroposterior diameters of the kidneys. This is greatest in the animals eating high protein in every case of the averages for the group. The nephrectomized animals that ate high protein diet had no kidney changes save hypertrophy and this amounted to an increase in weight of an average of 0.54 gm. or 85 per cent of the average weight of the right kidneys of the controls. Even on a diet of 40.13 per cent protein the limit of hypertrophy has not been reached as is shown by the results in the animals with one kidney.

So far at least, there is no proof of any damage to the kidneys of men who go from parts of the world where mixed diet is eaten to the far north and live entirely on meat. Stefansson (9) estimates that he has lived for a total of 9 years on an exclusive meat diet, the longest single period being 9 successive months. Lieb (10) has recently reviewed Stefansson's reports and writes, "Stefansson affirms that his observations on the health and longevity of the Eskimo led him to the conclusion that the high protein diet has no deleterious effect on their circulation or kidneys."

The work here reported contains no evidence for kidney damage resulting from high protein diet but the erroneous conclusion, that patients with nephritis may eat any amount of protein, must not be drawn. It is rational to relieve the diseased kidney of as much work as possible.

SUMMARY.

Seven groups of rats were fed on diets containing protein varying in amount from 1.36 to 40.13 per cent derived mainly from grain, casein, meat, and milk with carbohydrate, fat, and vitamines. From a number of animals one kidney was removed to double the load on the remaining one. The time of feeding was from 9 weeks to 6 months. Blood uric acid, blood urea nitrogen determinations, and microscopic examinations of the kidneys revealed no evidence of kidney damage. There was evidence of kidney hypertrophy consisting of increased

weight of the kidney, large diameters of the capillary tufts, convoluted tubules, and kidneys in the animals receiving high protein diet. The nephrectomized animals that ate high protein had no kidney changes save hypertrophy and this amounted to an increase in weight of an average of 0.54 gm. or 85 per cent of the average weight of the right kidneys of the controls.

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